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Urea Denaturation of Taka-amylase A

II. Kinetic Aspects

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(Received for publication, December 2, 1959)

In the previous paper (*1*) the effects of calcium and pH on the urea denaturation of taka-amylase A were examined. It was found that the viscosity of taka-amylase A solution in urea increased with time and reached a steady value within about 3000 minutes under the following condition: urea was added at pH 5.1 to taka-amylase A solution previously dialysed against distilled water. Under other condition no steady value of viscosity was obtained; even if a steady value were reached, it would take such a long time that additional effects might be accompanied. In order to treat the denaturation process quantitatively, it is necessary to find a steady state of denaturation. In the present work, therefore, taka-amylase A solution dialysed against water was treated with urea at pH 5.1 exclusively.

The change in viscosity depends on the over-all molecular shape and compactness, *i.e.*, the unfolding and aggregation. It has been confirmed, however, from the molecular weight determination by means of the measurements of sedimentation and diffusion that the aggregation is scarcely involved during the denaturation of taka-amylase A by urea under this condition (*2*). Further support for this has been provided by a preliminary observation that the optical rotation changes with time completely parallel to the viscosity.

The effects of urea concentration and temperature on the reaction rate and the final steady state of denaturation were examined extensively by the measurement of viscosity change which reflected the extent of molecular unfolding in this case, and then a possible reaction mechanism of denaturation was deduced to fit the experimental results obtained. Furthermore, the stability of taka-amylase A molecule against urea treatment was discussed in relation to the characteristic structure in the native state.

EXPERIMENTAL

Taka-amylase A and urea used in the present work were prepared and purified by the methods reported previously (*1*).

Taka-amylase A crystal was dissolved in distilled water and the solution was dialysed against water for 67 hours. Then 1 mole of this protein contains some definite amount of calcium between 1 to 10 moles (*1*). The protein solution and the acetate-buffered urea solution were brought to the reaction temperature mixed with each other and then the measurement of viscosity was immediately started. Prior to mixing, the protein solution had been diluted with water and the acetate-buffered urea solution had been prepared, in such a way as to adjust the concentrations of protein and urea, pH and ionic strength of the mixed protein-urea solution to the desired values. In most cases, the protein concentration in the mixture was about 0.5 per cent and the pH and ionic strength were adjusted to 5.1 and about 0.1, respectively. The protein and urea concentrations were determined in the same way as described previously (*1*).

The viscosity measurement was carried out according to the procedure described in the previous paper (*1*). The results of the viscosity experiments were expressed in terms of the kinematic viscosity, $(\tau-\tau_0)/\tau_0 c$, where τ is flow time of solution (protein-urea mixture), τ_0 that of solvent (urea solution of the same composition as the protein-urea solution, except that water has been added in place of the protein solution) and c the protein concentration expressed in g. per 100 ml.

RESULTS

Effect of Urea Concentration—The changes in viscosity with time for various urea concentrations at 20°C and 30°C are shown in Figs.

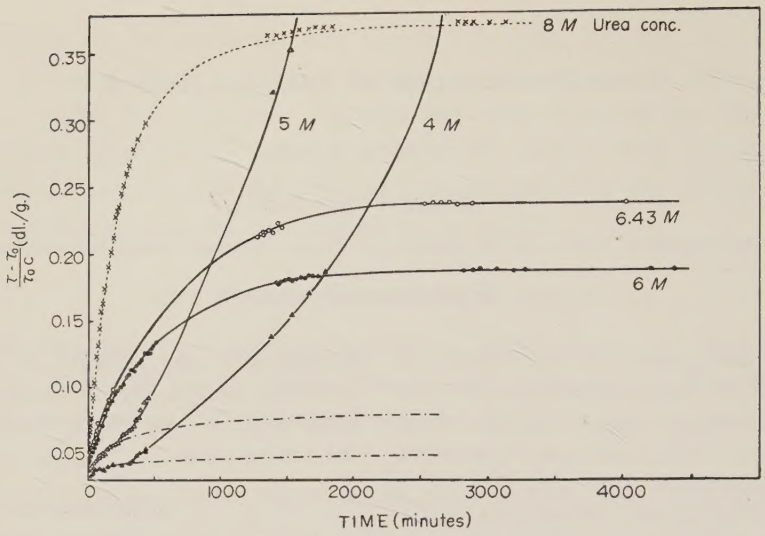

FIG. 1. Effect of urea concentration of the kinematic viscosity of 0.5% taka-amylase A at pH 5.1 (0.1 Γ/2 acetate buffer) at 20°C. Dashed curve for 8 *M* urea shows the theoretical curve calculated on the basis of the proposed denaturation mechanism.

1 and 2, respectively. At 20°C the viscosity of the solutions in which urea concentration was not less than 6 *M* reached a final steady value after increase with time, while the viscosity of the solutions in which urea concentration was less than 6 *M* had a tendency to settle in a steady value and then increased without limit, accompanied by the appearance of turbidity and precipitation. At 30°C the precipitation occurred in urea as high as 6 *M*. In an acetate buffer of pH 5.1 and ionic strength 0.1 but in the absence of urea, the viscosity of taka-amylase A solution did not change with temperature up to 30°C. It is inferred, therefore, that the taka-amylase A molecule which suffered some configurational chenge by urea is insoluble in urea solutions of the concentration lower than 6 *M* at 20°C and 6.43 *M* at 30°C. It is generally accepted that, when a protein is attacked by urea, intramolecular hydrogen bonds are ruptured and then new hydrogen bonds are formed between protein and urea, thus denatured protein remaining soluble in urea solution. If the interaction between denatured protein molecules are stronger than that between denatured protein and urea, aggregation takes place and finally leads to precipitation. Since the binding of protein with urea is exothermic,

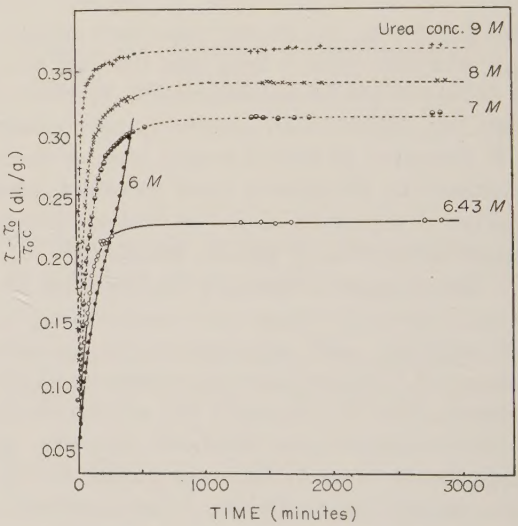

FIG. 2. Effect of urea concentration on the kinematic viscosity of 0.5% taka-amylase A at pH 5.1 (0.1 Γ/2 acetate buffer) at 30°C. Dashed curves for 7, 8 and 9 *M* urea show the theoretical curves calculated on the basis of the proposed denaturation mechanism.

the complex between urea and protein tends to dissociate at higher temperatures and greater urea concentrations are then required for its formation. Accordingly, at temperatures the denatured protein is soluble in urea solutions of lower concentrations, as the present

experimental results indicate.

It was observed that the precipitate separated out of the solution of denatured protein in urea when urea concentration was reduced by dialysis to some lower value, while the separated precipitate was redissolved when urea concentration was increased. The concentration range of urea in which the denatured protein remains dissolved appears to be consistent with the results shown in Figs. 1 and 2.

The effect of urea concentration on the final steady value of viscosity is shown in Fig. 3. As will be referred to later, the viscosity at the final state changed reversibly with temperature and was independent of temperature at which the protein had been denatured. For example, the viscosity value at 20°C of taka-amylase A denatured at 30°C was identical with that denatured at 20°C. Fig. 3 indicates values of final viscosity measured at 20°C and 30°C.

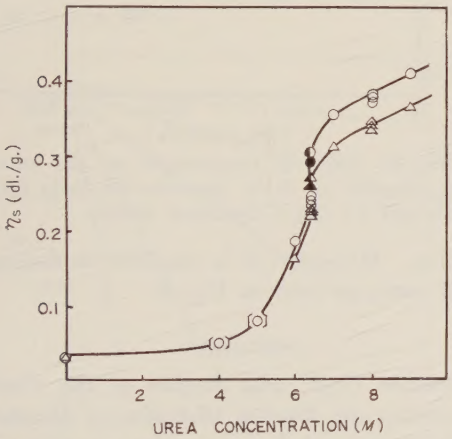

FIG. 3. Plots of the final steady value of kinematic viscosity against urea concentration at 20°C (circles) and 30°C (triangles). The values with [ ] for 4 *M* and 5 *M* urea represent the steady value obtained by extrapolation of the initial step of the viscosity-time curves, as shown by chain lines in Fig. 1.

Effect of Temperature—The changes in viscosity with time in 6.43 *M* urea at various temperatures from 20°C to 50°C are shown in Fig. 4 and those at 12°C and 16°C in Fig. 5. The changes in viscosity with time in 8 *M* urea at 20°C and 30°C are plotted in Fig. 6.

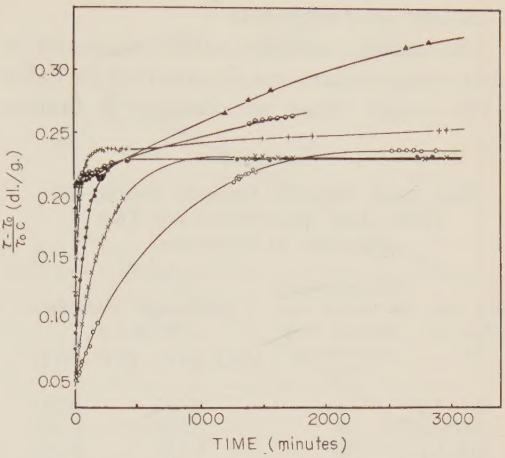

FIG. 4. Effect of temperature on the kinematic viscosity of 0.5% taka-amylase A in 6.43 *M* urea at pH 5.1 (0.1 Γ/2 acetate buffer). 20°C to 50°C.
—○— 20°, —×— 25°, —●— 30°, —+— 35°, —◒— 40°, —▲— 50°

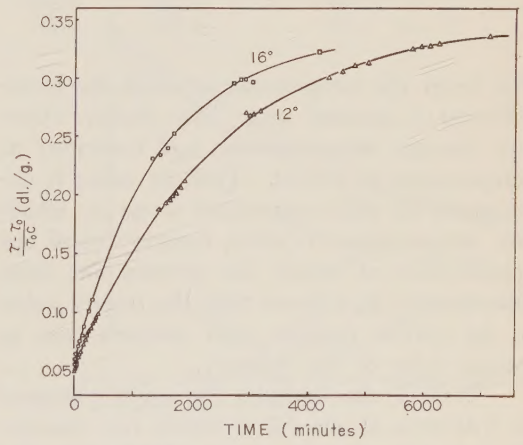

FIG. 5. Effect of temperature on the kinematic viscosity of 0.5% taka-amylase A in 6.43 *M* urea at pH 5.1 (0.1 Γ/2 acetate buffer). 12°C and 16°C.

At temperatures higher than 30°C, the viscosity still increases after reaching or approaching to a steady value, as shown in Fig. 4. This may be attributed to the formation of aggregates since the protein-protein interaction exceeds the protein-urea interaction as temperature is raised. The experimental results obtained from sedimentation and diffusion measurements (*2*) proved that the steady state of denaturation reflected in viscosity would

not include any aggregate.

The steady viscosity values measured at various temperatures are summarized in Table I. The steady value of viscosity is higher,

TABLE I

Final Steady Values of Kinematic Viscosity at Various Urea Concentrations and Their Dependence on Temperature

Urea concentration (M)	Temperature at which the protein was denatured (°C)	Kinematic viscosity (dl./g.) at 12°C	20°C	25°C	30°C
0 (pH 4.8)	—		0.031		0.031
6 (pH 7.2)	20		0.046		0.042
6 (pH 5.1)	20		0.187		0.164
6.43 (//)	12	0.349	0.307		0.272
6.43 (//)	20		0.237		0.231
6.43 (//)	25		0.242	0.231	0.227
6.43 (//)	30		0.248		0.230
6.44 (//)	20		0.291		0.261
7 (//)	30		0.356		0.314
8 (//)	20		0.382		0.348
8 (//)	20		0.372		0.338
8 (//)	30		0.383		0.342
9 (//)	30		0.412		0.367

the lower the temperature at which the measurement is carried out. The steady viscosity changes instantaneously and reversibly as temperature is varied. Thus its value is determined by the temperature alone at which the measurement is done, irrespective of the temperature at which the protein has been denatured.* It is found that the steady value of the optical rotation also behaves just as steady value of the viscosity.

Table I also includes the result obtained in 6 M urea at pH 7.2 at which the viscosity does not change with time at 20°C as described in the previous paper (*1*). In this case the temperature dependence of the viscosity value is very small. In 6.43 M urea at temperatures from 20°C to 35°C, the variation in final viscosity with temperature is relatively small and accordingly the final viscosities in Fig. 4 appear to be scarcely affected by temperature, the experimental error covering the variation. However, it is manifest in the case of 8 M urea, as seen in Fig. 6.

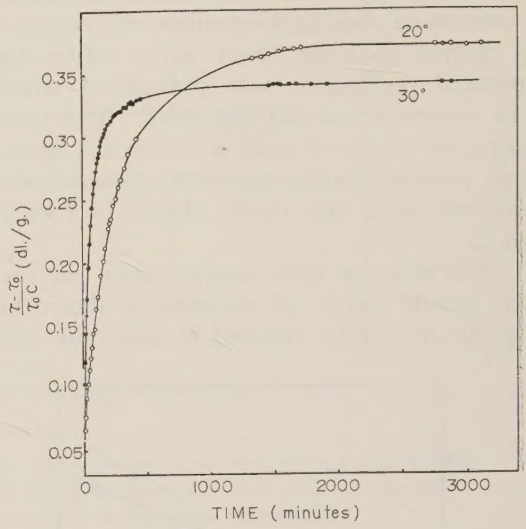

FIG. 6. Effect of temperature on the kinematic viscosity of 0.5% taka-amylase A in 8 M urea at pH 5.1 (0.1 $\Gamma/2$ acetate buffer).

DISCUSSION

Reaction Order with Respect to the Protein Concentration and Reaction Mechanism of Denaturation—It is assumed that the native taka-amylase A, N, is converted to the denatured one, S, by urea and the kinematic viscosity at time t is represented by a simple average:

$$\eta=\frac{[N]\eta_N+[S]\eta_S}{[N]+[S]}$$

where η_N and η_S are the kinematic viscosities of N and S, respectively, and [N] and [S] are the concentrations of N and S, respectively. If the reaction is of the first order with respect to [N], the following relation should be obeyed:

$$\eta_S-\eta=(\eta_S-\eta_N)\ e^{-kt}$$

where k represents the rate constant. The value of η_S is taken as the viscosity value at the steady state. The plots of log $(\eta_S-\eta)$ against time are shown in Figs. 7 to 11. For 6 M and 6.43 M urea straight lines are obtained at all temperatures examined, but for higher urea concentrations the plots fail to

* In 6.43 M urea a discrepancy exists between viscosity values measured at the same temperatare, for the protein denatured at 12°C (and 16°C) and other temperatures. Probably this comes from a minor difference in urea concentration, because the final value of viscosity is markedly influenced by the urea concentration near 6 M to 7 M, as clearly seen in Fig. 3.

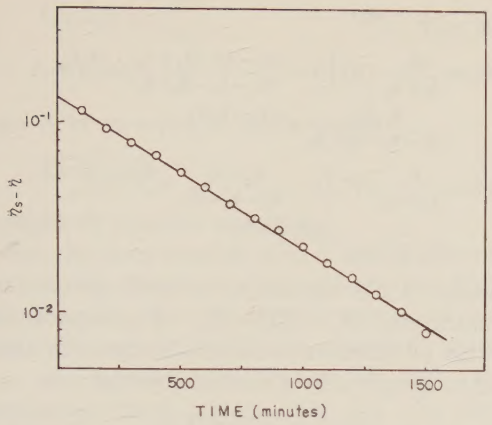

FIG. 7. Plot of log $(\eta_S - \eta)$ against time in 6 M urea at 20°C.

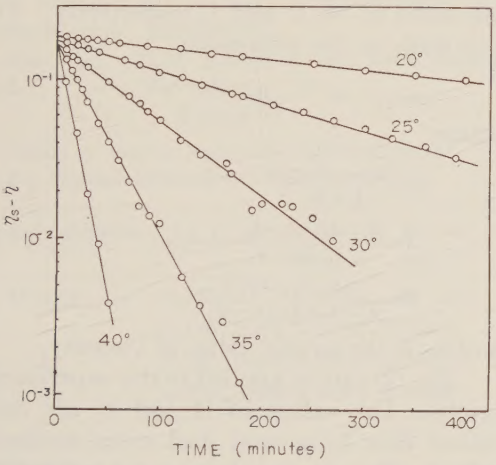

FIG. 8. Plots of log $(\eta_S - \eta)$ against time in 6.43 M urea at various temperatures from 20°C to 40°C.

give strainght lines, the reactions slowing down at their later stages.

The same kind of deviation from the first order reaction has been observed by Simpson and Kauzmann (3) in the urea denaturation of ovalbumin when a semilogarithmic plot of the change in optical rotation against time is done. These authors have listed several possible explanations to account for this. [1] The reaction is of a higher order; [2] a reactant other than protein is exhausted; [3] the product has an inhibiting effect on reaction; or [4] the process is a complex of simultaneous or successive first order reactions. They have adopted for the

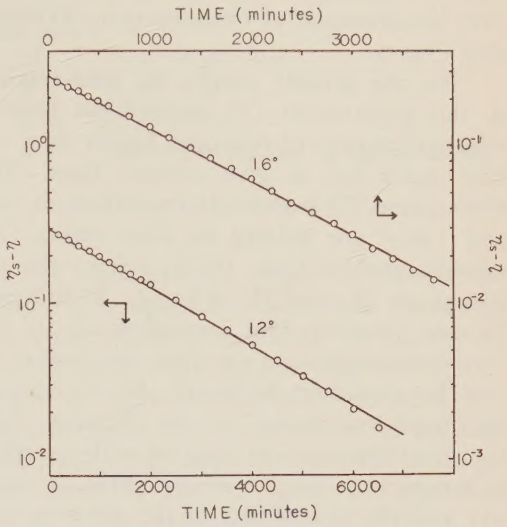

FIG. 9. Plots of log $(\eta_S - \eta)$ against time in 6.43 M urea at 12°C and 16°C.

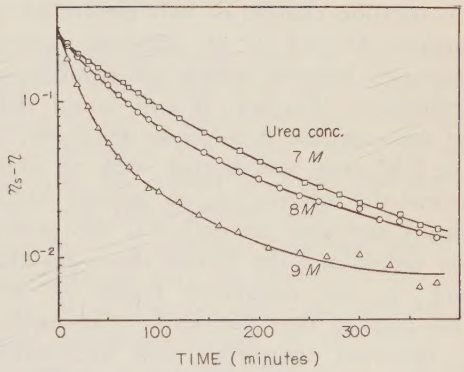

FIG. 10. Plots of log $(\eta_S - \eta)$ against time in 7, 8 and 9 M urea at 30°C.

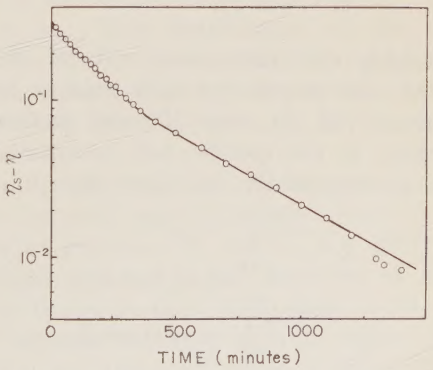

FIG. 11. Plot of log $(\eta_S - \eta)$ against time in 8 M urea at 20°C.

urea denaturation of ovalbumin the explanation [4].

For the present results on taka-amylase A the explanation [1] is ruled out because appropriate plots of reactions higher than the first order fail to give straight lines. The explanation [2] is also ruled out because urea and water are present in large excess. No experimental evidence which rejects the explanations [3] and [4] is found. It will now be seen, however, that instead of any of the above explanations a reaction mechanism as will be described below is able to account for the present results, *i.e.*, the deviation from the first order reaction together with the effect of temperature on the steady value of viscosity and the reversibility of the denaturation.

As already noted, the steady value of viscosity is a function of temperature. It is plausible to assume that the steady state of denaturation consists of two denatured components, D and I, of different kinematic viscosities, between which an equilibrium is established. Further, it was found that, if the taka-amylase A solutions denatured by 7, 8 or 9 M urea are diluted with or dialysed against the solvent, *i.e.*, an acetate buffer of pH 5.1 and ionic strength 0.1, the precipitation occurs and the filtrate shows no absorbance in an ultraviolet region at 280 mμ. This implies that the native taka-amylase A is converted completely and irreversibly to the denatured one. Then these observations lead to the reaction mechanism:

$$\mathrm{N} \xrightarrow{k_1} \mathrm{D} \underset{k_3}{\overset{k_2}{\rightleftharpoons}} \mathrm{I}$$

Assuming that each reaction is of the first order with respect to each protein concentration, [N], [D] and [I], and proceeds according to the specific rate constants, k_1, k_2 and k_3, respectively, it follows that

$$-\frac{d[\mathrm{N}]}{dt} = k_1[\mathrm{N}]$$

$$\frac{d[\mathrm{D}]}{dt} = k_1[\mathrm{N}]-k_2[\mathrm{D}]+k_3[\mathrm{I}]$$

$$\frac{d[\mathrm{I}]}{dt} = k_2[\mathrm{D}]-k_3[\mathrm{I}]$$

The solutions of these differential equations are

$$[\mathrm{N}]=[\mathrm{P}]\ \mathrm{e}^{-k_1 t}$$

$$[\mathrm{D}]=\frac{k_3}{k_2+k_3}[\mathrm{P}]\left\{1-\frac{(k_1-k_3)\ (k_2+k_3)}{(k_1-k_2-k_3)\ k_3}\ \mathrm{e}^{-k_1 t}+\frac{k_1\ k_2}{(k_1-k_2-k_3)\ k_3}\ \mathrm{e}^{-(k_2+k_3)t}\right\}$$

$$[\mathrm{I}]=\frac{k_2}{k_2+k_3}[\mathrm{P}]\left\{1+\frac{k_2+k_3}{k_1-k_2-k_3}\ \mathrm{e}^{-k_1 t}-\frac{k_1}{k_1-k_2-k_3}\ \mathrm{e}^{-(k_2+k_3)t}\right\}$$

where [P] is the initial or total protein concentration, [N]+[D]+[I]. The experimental value of kinematic viscosity at time t is assumed to be given by a simple average:

$$\eta=\frac{[\mathrm{N}]\eta_\mathrm{N}+[\mathrm{D}]\eta_\mathrm{D}+[\mathrm{I}]\eta_\mathrm{I}}{[\mathrm{N}]+[\mathrm{D}]+[\mathrm{I}]} \qquad (1)$$

where η_N, η_D and η_I represent the kinematic viscosities of N, D and I, respectively. Then this leads to the expression

$$\eta=\eta_\mathrm{S}-A\mathrm{e}^{-k_1 t}-B\mathrm{e}^{-(k_2+k_3)t} \qquad (2)$$

where

$$\eta_\mathrm{S}=\frac{k_2\eta_\mathrm{I}+k_3\eta_\mathrm{D}}{k_2+k_3} \qquad (3)$$

$$A=\frac{(k_1-k_3)\ \eta_\mathrm{D}-k_2\ \eta_\mathrm{I}}{k_1-k_2-k_3}-\eta_\mathrm{N} \qquad (4)$$

$$B=\frac{k_1 k_2\ (\eta_\mathrm{I}-\eta_\mathrm{D})}{(k_1-k_2-k_3)(k_2+k_3)} \qquad (5)$$

and η_S is the steady value of viscosity.

Eq. (2) can be applied to the experimental results. The values of A and k_1 are determined first by a trial and error method as follows. By plotting log $(\eta_\mathrm{S}-\eta)$ against t as shown in Figs. 10 and 11 are obtained the value of $\eta_\mathrm{S}-\eta_\mathrm{N}$ from the intersection at $t=0$ and the value of the initial rate constant, k, from the slope at small t's. Then the values of A and k_1 are determined so that the plot of log $(\eta_\mathrm{S}-\eta-A\mathrm{e}^{-k_1 t})$ against t is as straight as possible in the regions of $A<\eta_\mathrm{S}-\eta_\mathrm{N}$ and $k_1>k$, employing the observed values of η_S and η. From the intersection at $t=0$ and the slope of the straight line thus found, the values of B and k_2+k_3 are determined. The value of η_N is calculated from the relation $\eta_\mathrm{N}=\eta_\mathrm{S}-A-B$ (at $t=0$) and η_D from Eqs. (3) and (4). The values of η_I, k_2 and k_3 cannot be estimated directly, but they can be expressed by

$$\eta_\mathrm{I}=\frac{(1+K)\ \eta_\mathrm{S}-\eta_\mathrm{D}}{K}$$

$$k_2=(k_2+k_3)\frac{K}{1+K}$$

$$k_3=(k_2+k_3)\frac{1}{1+K}$$

where K is an equilibrium constant

$$K=\frac{[I]}{[D]}=\frac{k_2}{k_3}$$

althought it remains unknown.

For the denaturation in 7, 8 and 9 M urea in which the reactions deviate from the first order kinetics, the calculated values of all parameters together with the observed value of η_S are tabulated in Tables II and III. Substituting these values into Eq. (2), the theoretical viscosity-time curves are obtained, which are shown in Figs. 1 and 2. The agreement between theoretical and experimental curves is quite satisfactory.

On the other hand, it has been found that the denaturation obeys the first order kinetics in solutions in which urea concentration is not higher than 6.43 M. In this region, the identical reaction scheme may be likewise applied to the reaction system since the steady state still depends on temperature. If the conditions $k_2+k_3 \gg k_1$ and $k_3 \gg k_1$ are satisfied, the third term in Eq. (2) may be neglected and A is approximated by the relation $A=\eta_S-\eta_N$. Then Eq. (2) leads to a simpler expression

$$\eta_S-\eta = (\eta_S-\eta_N)\, e^{-k_1 t}$$

which implies the linear plot of log $(\eta_S-\eta)$ against t. The values of k_1 and A and consequently η_N are obtained from the slope and the intersection of this line. Tables II and III include the values of these parameters together with the observed value of η_S.

For the urea concentrations not less than 7 M it was found that the value of k_2+k_3 was scarcely dependent on urea concentration at constant temperature and of the order of magnitude of about one tenth of k_1. On the other hand, for the urea concentrations not more than 6.43 M, it was assumed that the values of k_2+k_3 and k_3 are much larger than that of k_1. This discontinuity of the rate constants in the region near 6 to 7 M urea may have some relation to the abrupt change in the final viscosity value, η_S, in the same region.

In the present stage of investigation, it cannot be said how the actual states of the denatured proteins, D and I, are different from each other. Larger amounts of urea may be bound to the I form than to the D form, because the value of η_I is higher than that of η_D and the equilibrium is shifted from D to I as the temperature is lowered. How-

TABLE II

Values of Various Parameters Calculated for the Proposed Denaturation Mechanism (30°C)

Urea concentration M	A	B	k_1 min.$^{-1}$	k_2+k_3 min.$^{-1}$
6.43	0.170		0.0113	$\gg k_1$[1]
7	0.180	0.074	0.0140	0.00431
8	0.220	0.070	0.0230	0.00435
9	0.270	0.036	0.0470	0.00467

Urea concentration M	η_S dl./g.	η_N dl./g.	η_D dl./g.	η_I dl./g.
6.43	0.230	0.060	—	—
7	0.314	0.060	0.263	$0.051/K+0.314$
8	0.341	0.051	0.284	$0.057/K+0.341$
9	0.367	0.061	0.335	$0.032/K+0.367$

1) In 6.43 M urea, $k_3 \gg k_1$.

TABLE III

Values of Various Parameters Calcuatled for the Proposed Denaturation Mechanism (20°C)

Urea concentration M	A	B	k_1 min.$^{-1}$	k_2+k_3 min.$^{-1}$
6	0.140	—	0.00171	$\gg k_1$[1]
6.43	0.190	—	0.00168	$\gg k_1$[1]
8	0.200	0.120	0.00570	0.00174

Urea concentration M	η_S dl./g.	η_N dl./g.	η_D dl./g.	η_I dl./g.
6	0.187	0.047	—	—
6.43	0.237	0.047	—	—
8	0.372	0.052	0.288	$0.083/K+0.372$

1) In 6 M and 6.43 M urea, $k_3 \gg k_1$.

ever, I is not an aggregate of D, as will be shown in the succeeding paper (*2*).

It is noticed that the values of η_N, *i.e.*, the initial kinematic viscosity in the urea denaturation, are only slightly higher than the value of kinematic viscosity of the native protein in an acetate buffer, *i.e.*, 0.031 dl. per g. (*4*). It is likely that no immediate increase in viscosity takes place after adding urea, if the error of measurement and difference in conditions are taken into account.

It is found that the value of η_D at constant temperature is larger in more concentrated urea solutions but it is scarcely influenced by temperature at constant urea concentration. This indicates that the state of the component D is only dependent on urea concentration but not on temperature. Hence, the temperature dependence of the final steady viscosity, η_S, can be ascribed to that of the equilibrium constant, K, if η_I may be also assumed to be independent of temperature.

The temperature dependence of η_S can be explained by a solvent effect that a denatured protein molecule is more swollen and has a higher viscosity value owing to the stronger protein-urea interaction at lower temperatures. However, the deviation from the first order kinetics as well as the temperature dependence of η_S should be interpreted preferably on the same ground. Then it might be more suitable to account for the present experimental results in terms of the reaction mechanism as described above rather than in terms of the simple solvent effect. This denaturation mechanism may not be exclusive, but will be a very plausible one since it can be applied successfully to the present data, although it involves an essential assumption of the additivity of viscosity values as formulated in Eq. (1).

Some possible mechanisms of denaturation have been presented so far. These are classified in the following two schemes:

$$[1]\quad \text{Native protein} \rightleftharpoons \text{Reversibly denatured protein} \longrightarrow \text{Irreversibly denatured protein}$$

$$[2]\quad \text{Irreversibly denatured protein} \longleftarrow \text{Native protein} \rightleftharpoons \text{Reversibly ``protected'' protein}$$

The first mechanism is that postulated by Lundgren and Williams (*5*) for the denaturation of thyroglobulin, by Christensen (*6*, *7*) for the urea denaturation of β-lactoglobulin and by Simpson and Kauzmann (*3*) for the urea denaturation of ovalbumin. The second mechanism has been postulated by Wright and Schomaker (*8*, *9*) for the for inactivation of antibodies, notably diphteria antitoxin by urea, by Chase (*10*) for the heat inactivation of luciferase and by Foster and Yang (*11*) for the heat denaturation of plasma albumin at low pH. The reaction mechanism presented here for the urea denaturation of taka-amlyase A does not belong to any of those so far presented.

Reaction Order with Respect to Urea Concentration—The rate constant of the first step of denaturation, k_1, is strongly dependent on urea concentration, as seen from Tables II and III. If the first order rate constant is expressed by

$$k_1 = k_1' [U]^n$$

where [U] is the urea concentration, strictly speaking, the thermodyamic activity of urea, and k'_1 is a constant independent of urea concentration at constant temperature, then the plot of log k_1 against log [U] will give a straight line with a slope equivalent to n, *i.e.*, the reaction order with respect to urea concentration. In fact, this plot has given a straight line for the urea denaturation of tobacco mosaic virus (*12*) and ovalbumin (*3*, *13*). For ovalbumin Kauzmann has found $n=15$ at 0°C and has concluded that the simultaneous attack on the protein by 15 or more molecules of urea is necessary to cause the unfolding of the ovalbumin molecule at 0°C, that is, the folds in the polypeptide chain cannot be opened unless several hydrogen bonds are broken simultaneously.

For the urea denaturation of taka-amylase A, on the other hand, the plot of log k_1 against log [U] does not give a straight line at both temperatures, 20°C and 30°C, as shown in Fig. 12. In this case, therefore, the same explanation as above does not hold and one must consider more complicated factors de-

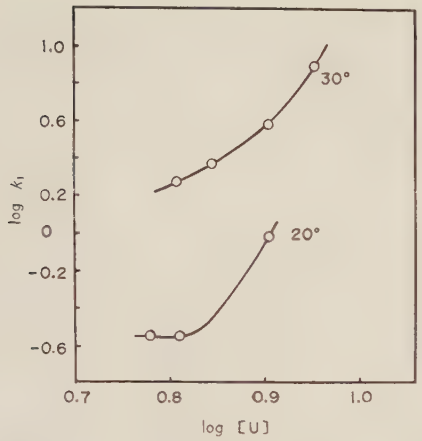

FIG. 12. Dependence of the rate constant of the first step of denaturation on urea concentration at 20°C and 30°C. Plots of log k_1 *vs.* log [U].

pending on urea concentration. It was found that the rate constant, k_1, is constant irrespective of urea concentration in the region where the denaturation obeys the first order behavior, whereas it increases with urea concentration in the region where the denaturation deviates from the first order behavior. The increasing rate of k_1 with urea concentration is smaller at higher temperatures, as in the cases of tobacco mosaic virus (*12*), ovalbumin (*3*) and fibrinogen (*14*).

Effect of Temperature on the Rate Constant—Lauffer (*12*) found that the temperature coefficient of the denaturation of tobacco mosaic virus in urea was negative below room temperature and positive above it. Simpson and Kauzmann (*3*) also showed strikingly similar behavior for the urea denaturation of ovalbumin as measured by optical rotation. (Hopkins (*15*) reported that the urea denaturation of ovalbumin had a negative temperature coefficient between 0°C and 37°C, deducing from a relatively rough measurement by the isoelectric precipitation method.) Jacobsen and Christensen (6, 7) found that the rate constant of the urea denaturation of β-lactoglobulin had a minimum at 30°C. Hopkins, Lauffer, Christensen and Simpson and Kauzmann explained the unusual effect of temperature on the rate by assuming the reaction scheme which was described in the previous section as the mechanism [1]. In this mechanism, the second step is faster but the reverse reaction of the first step is accelerated, as the temperature is raised, and, therefore, a minimum rate appears.

On the other hand, the urea inactivation of diphteria antioxion studied by Wright and Schomaker (*8*) had an overall positive temperature coefficient through the region of 0°C to 40°C. For this inactivation they postulated the reaction scheme written as the [2] in the previous section. Mihalyi (*14*) found that for the urea denaturation of fibrinogen an increase in temperature brought about an increase in the reaction rate over 10°C to 45°C.

For taka-amylase A the rate of increase in viscosity during the urea denaturation showed a positive temperature coefficient over the region of 12°C to 50°C, as shown in Figs. 4, 5 and 6. Fig. 13 shows the Arrhenius

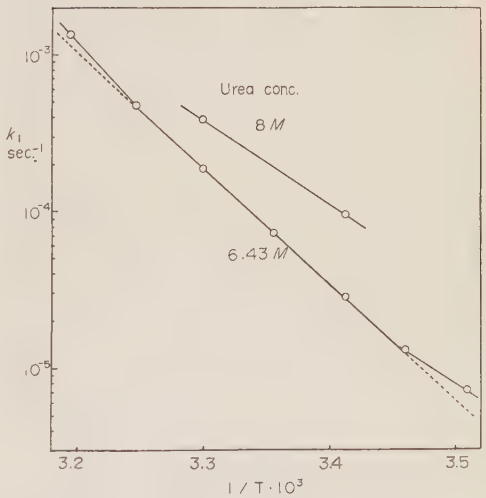

FIG. 13. Arrhenius plots of the rate constants of the first step of denaturation in 6.43 *M* and 8 *M* urea.

plot of the results, *i.e.*, the logarithm of the rate constant, k_1, of the first step of the present reaction scheme plotted against the reciprocal of the absolute temperature. At 6.43 *M* urea concentration, the Arrhenius plot gives a straight line between 16°C and 35°C, but above and below these temperature

region there are deviations from this line. The Arrhenius activation energy, E, can be estimated from the slope of this line. Further, the heat of activation, ΔH^*, the free energy of activation, ΔF^*, and the entropy of activation, ΔS^*, are calculated by the Eyring's equations

$$\Delta H^* = E - RT$$

$$\Delta F^* = RT\ (\ln \frac{k}{h} + \ln T - \ln k_1)$$

$$\Delta S^* = \frac{\Delta H^* - \Delta F^*}{T}$$

where k is the Boltzmann constant and h is the Planck constant. These calculated values are summarized in Table IV. At con-

TABLE IV

The Arrhenius Activation Energy, and the Heat, the Free Energy and the Entropy of Activation in the Urea Denaturation of Taka-amylase A

Urea concentration M	Temperature °C	E cal./mole	ΔH^* cal./mole	ΔF^* cal./mole	ΔS^* e.u.
6.43	12—16	24200	23600	23400	0.70 (12°C)
	16—35	33750	33200	23200	34.1 (20°C)
	35—40	39500	38900	22500	52.4 (40°C)
8	20—30	24500	23900	22500	4.78 (20°C)

urea concentration ΔH^* and ΔS^* have larger values at higher temperatures, and at constant temperature they have smaller values at higher urea concentrations. This aspect is strikingly similar to that observed by Mihalyi (*14*) in the urea denaturation of fibrinogen. It may be explained after him. The activation process of urea denaturation involves the rupture of intramolecular hydrogen bonds accompanying the combination of urea melcules. The binding of urea molecules is an exothermic reaction, and the heat liberated compensates partly the heat consumed during the activation process. At the same time, the entropy of the activation process might be reduced because the groups bound with urea should be somewhat frozen-in. At lower temperatures or at higher urea concentrations the number of bound urea molecules is increased, and, therefore, the heat and entropy of activation should be reduced.

The Stability of Taka-amylase A Molecule— In contrast to the urea denaturation of serum albumin, in which the changes in optical rotation and viscosity took place almost instantaneously and reversibly at all urea concentrations (*16*), the viscosity of taka-amylase A increased relatively slowly with time by urea denaturation. Since the viscosity change of taka-amylase A in urea does not involve molecular aggregation but only molecular unfolding (*2*), it can be implied that the rate of unfolding of the taka-amylase A molecule is relatively slow in urea. On the other hand, taka-amylase A was readily denatured even in urea solutions as dilute as 2 M. Under this condition the solution became turbid immediately after adding urea, indicat ing that a molecular change occurs to make the protein insoluble. Moreover, taka-amylase A did not return to the original state on removing or diluting urea. Thus, according to the terminology of Kauzmann (*17*), native taka-amylase A has a high "kinetic stability" but a low "thermodynamic stability" and belongs to a protein of the "Type II". It may be supposed that taka-amylase A is similar to ovalbumin in its stability against urea denaturation. The slow and irreversible denaturation of taka-amylase A suggests that the molecule is relatively rigid and compact, as compared with proteins such as serum albumin. This conclusion is consistent with that derived from the hydrodynamic studies of native taka-amylase A (*18*).

From the inference described in the previous paper (*1*), it can be concluded that the tertiary structure including intramolecular calcium salt linkages contributes greatly to the kinetic stability of taka-amylase A. An evidence that the tertiary structure of taka-amylase A is relatively strong is given by the slow denaturation when it is treated with sodium dodecyl sulfate (*19*), which would be responsible to weaken the tertiary structure.

SUMMARY

1. Effects of urea concentration and temperature on urea denaturation of taka-amylase A were examined by the measure-

ment of viscosity. The experiment was carried out for taka-amylase A solution prepared by dialysis against water. When this solution was treated with urea in an acetate buffer of pH 5.1 and ionic strength 0.1, the viscosity reached a steady value after it increased with time.

2. The final steady value of viscosity increased with urea concentration and changed abruptly in the region of 6 M to 7 M urea.

3. The steady value of viscosity was determined only by the temperature at which the viscosity was measured, irrespective of the temperature at which the protein had been denatured. It was higher as the temperature was lower.

4. If the taka-amylase A solution denatured by concentrated urea solutions was diluted with or dialysed against the buffer solution, precipitation occurred and filtrate showed no absorbance in an ultraviolet region at 280 mμ.

5. The denaturation obeyed the first order reaction with respect to the protein concentration in solutions in which the urea concentration was not higher than 6.43 M, whereas it deviated from the first order kinetics at higher urea concentrations.

6. A possible reaction mechanism of denaturation was deduced to fit all these experimental results. It may be expressed by the scheme

$$\text{Native protein} \xrightarrow{k_1} \text{Denatured component 1} \underset{k_3}{\overset{k_2}{\rightleftharpoons}} \text{Denatured component 2}$$

7. The rate constant of the first step of denaturation, k_1, was dependent on urea concentration.

8. The denaturation rate had a positive temperature coefficient over the region of 12°C to 50°C. The Arrhenius activation energy, and the heat, the free energy and the entropy of activation at different urea concentrations and temperatures were estimated.

9. The unfolding of taka-amylase A molecule by urea treatment proceeded with a relatively slow rate, but the protein was readily denatured even in urea solution of low concentration. Consequently, taka-amylase A has a high "kinetic stability" but a low "thermodynamic stability".

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REFERENCES

(1) Fujita-Ikeda, S., and Isemura, T., *J. Biochem.*, **47**, 537 (1960)
(2) Isemura, T., and Fujita-Ikeda, S., *J. Biochem.*, **49**, 278 (1961)
(3) Simpson, R.B., and Kauzmann, W., *J. Am. Chem. Soc.*, **75**, 5139 (1953)
(4) Isemura, T., and Fujita, S., *J. Biochem.*, **44**, 443 (1957)
(5) Williams, J., and Lundgren, H., *J. Phys. Chem.*, **43**, 989 (1939)
(6) Jacobsen, C.F., and Christensen, L.K., *Nature*, **161**, 30 (1948)
(7) Christensen, L.K., *Compt. rend. trab. lab. Carlsberg, Sér. Chim.*, **28**, 39 (1952)
(8) Wright, G.G., and Schomaker, V., *J. Am. Chem. Soc.*, **70**, 356 (1948)
(9) Wright, G,G., and Schomaker, V., *J. Biol. Chem.*, **175**, 169 (1948)
(10) Chase, A.M., *J. Gen. Physiol.*, **33**, 535 (1950)
(11) Foster, J.F., and Yang, J.T., *J. Am. Chem. Soc.*, **77**, 3895 (1955)
(13) Lauffer, M.A., *J. Am. Chem. Soc.*, **65**, 1793 (1943)
(13) Kauzmann, W., "A Symposium on the Mechanism of Enzyme Action," ed. by McElroy, W. D., and Glas, B., Johns Hopkins University, p. 70 (1954)
(14) Mihalyi, E., *Acta Chem. Scand.*, **4**, 317 (1950)
(15) Hopkins, F.G., *Nature*, **126**, 328, 383 (1930)
(16) Kauzmann, W., and Simpson, R.B., *J. Am. Chem., Soc.*, **75**, 5154 (1953)
(17) Kauzmann, W., *J. Cell Comp. Physiol.* **47**, Supplement 1, 113 (1956)
(18) Isemura, T., and Fujita, S., *J. Biochem.*, **44**, 797 (1957)
(19) Isemura, T., and Takagi, T., *J. Biochem.*, **46**, 1637 (1959)

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Urea Denaturation of Taka-amylase A

III. Size and Shape of Denatured Protein Molecule

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In the previous papers (*1*, *2*) the kinetics of urea denaturation of taka-amylase A was studied mainly by viscosity measurements. It was observed that the kinematic viscosity of taka-amylase A in urea solution increased with time and the mode of this change was profoundly affected by experimental conditions, *i.e.*, by the amount of calcium bound to the protein, pH, temperature and urea concentration. Under suitable conditions the viscosities were found to reach steady values. A change in the viscosity of a protein indicates a change in the over-all molecular shape and compactness. Accordingly, the steady viscosity value attatined suggests that the denatured protein molecule assumes a certain definite configuration. It is desirable to investigate in more detail the structural change of the protein molecule upon denaturation. Previously the sedimentation, diffusion and intrinsic viscosity of native taka-amylase A were measured (*3*). In the present work these hydrodynamic properties of denatured taka-amylase A are measured under conditions when the viscosity of the protein in urea solution has ceased to increase with time. The results on the molecular size and shape of taka-amylase A denatured by urea are compared with those on the native protein.

EXPERIMENTAL

Taka-amylase A and urea used in the present investigation were prepared and purified as previously described (*1*).

To obtain the steady state of denaturation, taka-amylase A to which a definite amount of calcium is bound must be used. The amount of bound calcium varies with the method of dialysis used to prepare the protein solution. A definite pH and temperature range on denaturation reaction must also be chosen. These experimental conditions, as well as the methods of preparing the protein and urea solutions and of mixing them were as follows:

The taka-amylase A solution was prepared by dissolving the crystalline protein and dialysing it against distilled water. It was mixed with the urea solution in acetate buffer at 30°C. The mixture was of pH 5.1 and ionic strength 0.1. When the viscosity had ceased to increase with time, the intrinsic viscosity and sedimentation and diffusion coefficients were measured. The concentration of protein in the urea solution was about 0.5 per cent in each experiment.

The viscosity was measured with an Ostwald viscometer having a flow time of 120 to 180 seconds for water. To measure the intrinsic viscosity of the denatured protein solution, the solution was succesively diluted with the solvent (a solution of the same composition as the protein-urea-buffer mixture, except that water has been added in place of the protein solution), and the viscosity of the solution at each dilution was measured. The reduced viscosity values were extrapolated graphically to infinite dilution.

The sedimentation experiment was performed in a Spinco Model E Ultracentrifuge at 59,780 r.p.m.. The diffusion coefficient was measured at 20°C with a Hitachi Model HT-B electrophoresis apparatus combined with a Neurath type diffusion cell. The solution was diffused against the solvent, *i. e.*, a urea solution, against which the former had previously been dialysed. The density of the solution and solvent was measured using an Ostwald picnometer with a capacity of about 3 ml.

RESULTS

The reduced viscosity of denatured taka-amylase A solution is plotted against protein concentration in Fig. 1. The figure also includes results for the native protein in acetate buffer, pH 4.8 (*3*, *2*) and for the protein treated with 6 *M* urea at 20°C in veronal buffer, pH 7.2 (*1*, *2*). In this last, the viscosity did not

increase with time but its value was slightly higher than for the protein in acetate buffer only (*1*).

The sedimentation coefficient was measured with 0.5 per cent taka-amylase A solutions denatured in 7.8 and 9 *M* urea, respectively. A series of sedimentation patterns is illustrated in Fig. 2. In each experiment one peak only

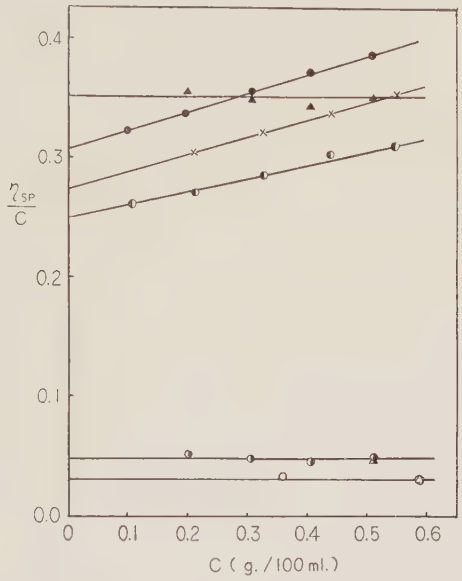

Fig. 1. Plots of reduced viscosity against protein concentration. ○, △, for native taka-amylase A; ◑, ◭, for taka-amylase A treated with 6 *M* urea at 20°C, in veronal buffer, pH 7.2; ×, ◐ and ●, ▲, for taka-amylase A denatured by 6.43 *M* and 8 *M* urea, respectively, in acetate buffer, pH 5.1. Crosses, circles and triangles show the values measured at 12°, 20° and 30°C, respectively.

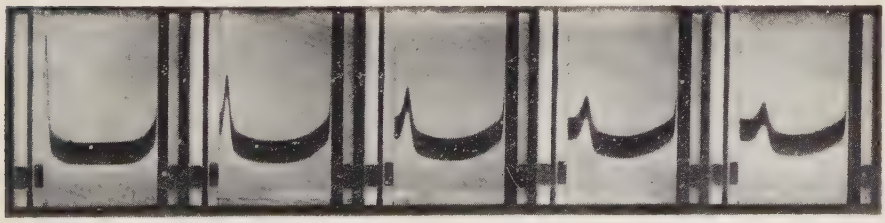

Fig. 2. Sedimentation diagrams of 0.5% taka-amylase A in 8 *M* urea at pH 5.1 (measured at 27.2°C). Photographs were taken at intervals of 32 minutes at a rotor speed of 59,780 r.p.m..

Table I

Diffusion and Sedimentation Coefficients and Molecular Weights of Native and Urea-denatured Taka-amylase A

Urea concentration *M*	$D_{A(20,w)}$	$D_{M(20,w)}$	$D_{0(20,w)}$ $\times 10^7$ *cm²./sec.*	D_M/D_A	$s^{20,w}$ *S*	*M*
0 (native)	7.37	7.37	7.39	1	4.67	51,000
7	—	—	—	—	2.88	—
8	3.69	4.03	3.40	1.09	2.81	57,000
9	3.59	3.91	3.26	1.09	2.33	48,000

was observed. The values of the sedimentation coefficient reduced to those in water at 20° are shown in Table I.

The diffusion coefficient was measured with 0.5 per cent taka-amylase A solutions denatured in 8 *M* and 9 *M* urea, respectively. The values of diffusion coefficients reduced to those in water at 20° are summarized in Table I, in which D_A is that calculated by the area method and D_M that calculated by the statistical method. It can be seen that the latter is slightly larger than the former*, which means that the denatured protein molecule exhibits some polydispersity with respect to the diffusion coefficient (*4*). The concentration gradient curve was somewhat asymmetric, the maximum ordinate shifting towards the side of the solvent*. This implies that the diffusion coefficient of the denatured protein depends to some extent on the protein concentration. Since this dependence was not examined experimentally, the values of the diffusion coefficients at infinite dilution, D_0, were estimated by the Gralén method (*5*). These values are also shown in Table I.

DISCUSSION

Dependence of the Reduced Viscosity on the Protein Concentration—The reduced viscosities of native taka-amylase A and of taka-amylase A treated with 6 *M* urea at pH 7.2 and 20° were not dependent on the protein concentration and there was no difference between values measured at 20° and 30°. However the reduced viscosity of a denatured protein solution, which was much greater, was dependent on the protein concentration when diluted after denaturation. This dependence was larger at lower temperatures. In accordance with the mechanism of denaturation kinetics proposed in the preceding paper (*2*), the denatured protein may be assumed to consist of two components, named D and I, which are in equilibrium. The proportion of I is higher at lower temperatures. That is, the equilibrium is shifted from D to I. If it is assumed that the concentration dependence of the reduced viscosity of I is larger than that of D, then the change in viscosity of the denatured protein may be adequately explained. Apart from the denaturation mechanism however, there is an alternative explanation. It is generally observed that a flexible chain polymer in a good solvent has a lower slope of the reduced viscosity-concentration curve at higher temperatures (*6*, *7*). If concentrated urea is regarded as a solvent for the denatured protein, the same would apply here, because the denatured protein would have a greater flexibility than the native one.

Molecular Weight and Shape of the Denatured Protein Molecule—It can be seen from Table I that the sedimentation and diffusion coefficients of denatured protein are less than those of the native one and they are reduced as the intrinsic viscosity increases, *i.e.*, as denaturation proceeds. Whereas the results on viscosity suggested the formation of two components, D and I, in the denatured protein (*2*), the sedimentation pattern shows only one peak. However, the concentration gradient curve of diffusion indicates some polydispersity of the denatured protein. Accordingly the single peak of the sedimentation pattern might be attributed to a rapidly attained equilibrium between the two components (*8*), to an insignificant difference in their sedimentation velocities, or to Johnston-Ogston's effect (*9*). It seems probable that the conversion of D to I involves neither association nor dissociation, since under a certain condition the reduced viscosity of the denatured protein, *i.e.*, of a mixture of D and I, showed no dependence on the protein concentration.

By the use of the Svedberg equation,

$$M = \frac{NkTs}{D\,(1-\bar{v}\rho_w)}$$

where N is the Avogadro number, k the Boltzmann constant, T the absolute temperature and ρ_w the density of water, the values of the sedimentation coefficient, $s_{20,w}$, and diffusion coefficient, $D_{M\,(20,w)}$, given in Table I lead to molecular weights of 57,000 and 48,000 in 8 *M* and 9 *M* urea, respectively, when the partial specific volume, $\bar{v}$, of the denatured protein is assumed to be equal to

* The concentration gradient curve of native taka-amylase A was found to be symmetric and Gaussian (*3*).

that of the native protein, 0.700 ml. per g. (*3*). Taking into account the errors involved in the use of values not extrapolated to infinite dilution and of the dubious value for the partial specific volume, the calculated molecular weights must be only approximate*. However, in comparing these molecular weights with that of the native protein, 51,000 (*3*), it seems likely that the urea denaturation of taka-amylase A causes neither molecular association nor dissociation. The fact that no association takes place is consistent with the result that denaturation is a first order reaction with respect to the protein concentration (*2*).

From these considerations, the increase in viscosity or decrease in sedimentation and diffusion coefficients upon urea denaturation can be attributed exclusively to configurational changes in the protein molecule itself, *i.e.*, an increase in asymmetry or in volume of the hydrodynamically equivalent ellipsoid. According to Scheraga and Mandelkern (*12*), a shape factor, β, can be estimated by the formula:

$$\beta = \frac{Ns\,[\eta]^{1/3}\,\eta_w}{M^{2/3}\,(1-\bar{v}\rho_w)} \qquad (1)$$

$$\beta = \frac{D[\eta]^{1/3}M^{1/3}\eta_w}{kT} \qquad (2)$$

where $[\eta]$ is the intrinsic viscosity of the solution and η_w the viscosity of water. To calculate β by Eq. (1) the partial specific volume, $\bar{v}$, must be known. However, it is difficult to measure this accurately for urea-denatured taka-amylase A since the denatured protein is obtained after so long treatment with urea that the density of the urea in solution has also changed. The β value was, therefore, calculated exclusively by Eq. (2). Assuming the molecular weight of the urea-denatured taka-amylase A to be 51,000 and using D_0 calculated by the Gralén method, the β factor was found to have the values given in Table II. It can be seen that the β factor of the denatured taka-amylase A scarcely differs from that of the native protein. Therefore the increase in viscosity and decrease in sedimentation and diffusion coefficients must be ascribed, not to a change in shape of the hydrodynamically equivalent ellipsoid, but to a change in its effective volume, V_e. In such a case the following relationships should be fulfilled:

$$\frac{(V_e)_{denat.}}{(V_e)_{nat.}} = \frac{[\eta]_{denat.}}{[\eta]_{nat.}} \qquad (3)$$

$$= \frac{D^3_{nat.}}{D^3_{denat.}} \qquad (4)$$

Table II

Shape Factors and Hydrodynamically Effective Volumes of Native and Urea-denatured Taka-amylase A

Urea concentration M	$D_0(_{20,\,w}) \times 10^7$ $cm^2./sec.$	$[\eta]$ $dl./g.$	$\beta \times 10^{-6}$	$\frac{V_{e,den\,t.}}{V_{e,nat.}}$ $\frac{[\eta]_{denat.}}{[\eta]_{nat.}}$	$\frac{D^3_{nat.}}{D^3_{denat}}$
0 (native)	7.37	0.034	2.21	—	—
8	3.40	0.310	2.13	9.12	10.2
9	3.26	0.328	2.11	10.0	11.5

The ratio of the effective volume of denatured to native taka-amylase A is also shown in Table II. The values derived from Eqs. (3) and (4) respectively, are nearly identical. Thus denaturation in 8 M or 9 M urea increases the effective volume ten times. Cases when the β value remains unchanged but the V_e value increases with denaturation are found in the urea denaturation of serum albumin (*12*) and fibrinogen (*13*). These are in contrast to the

* On applying the formula for the concentration dependence of the sedimentation coefficient deduced from Burger's theory (*10*), the values of the sedimentation coefficient, s_0, at infinite dilution can be found. From the values of s_0 and D_0, the molecular weight is calculated as about 70,000 in both 8M and 9M urea. Since the partial specific volume of denatured protein is generally smaller than that of the native protein (*11*), the molecular weight must be lower than this.

alkali denaturation of pepsin (*14*) when the β value increases but the V_e value is not changed. However, the increase in effective volume does not necessarily mean an actual swelling of the molecule. A preliminary study of the optical rotation showed that levo-rotation of taka-amylase A increased as the denaturation by urea proceeded and was in parallel with increase in viscosity. An increase in levo-rotation can be attributed to the conversion of a polypeptide chain from a helical form to a random coil (*15*). Thus it is more likely that the taka-amylase A molecule is converted from a rigid configuration to some loose configuration such as a random coil, by unfolding of the polypeptide chain. This results in higher viscosity and lower sedimentation and diffusion coefficients.

If the intrinsic viscosities of taka-amylase A in 8 M and 9 M urea, 0.310 and 0.340 dl. per g., respectively, are compared with that of a linear polymer of the same molecular weight in a good solvent, *e.g.*, that of polyisobutylene of molecular weight 50,000 in cyclohexane, 0.47 dl. per g. (*16*), it is found that the denatured taka-amylase A is still far from a random coil. This is also supported by the value obtained for β, which is less than 2.5 for random coil molecules. Taka-amylase A contains four or five disulfide bridges in the molecule (*17*). The cross linkages which remain unruptured under the present experimental conditions will be responsible for preventing the molecule from becoming a random coil.

SUMMARY

1. To obtain information on the molecular size and shape of taka-amylase A denatured by urea, sedimentation, diffusion and viscosity were measured under conditions when the viscosity had ceased to increase with time. A taka-amylase A solution, prepared by dialysis against water, was treated with urea in acetate buffer, pH 5.1, ionic strength 0.1.

2. When the protein solution was successively diluted after denaturation, the dependence of the reduced viscosity of the denatured protein on the protein concentration was larger at lower temperatures.

3. The intrinsic viscosity increased and the sedimentation and diffusion coefficients decreased as denaturation proceeded.

4. The molecular weight of the denatured protein, calculated from the sedimentation and diffusion coefficients, when allowance was made for experimental uncertainties, agreed with that of the native protein.

5. A shape factor, β, of the denatured protein, estimated according to the method of Scheraga and Mandelkern, scarcely differed from that of the native protein. Thus the changes of hydrodynamic properties by urea denaturation were ascribed to an increase in the hydrodynamically effective volume. In 8 M to 9 M urea this was ten times larger than before denaturation.

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REFERENCES

(*1*) Fujita-Ikeda, S., and Isemura, T., *J. Biochem.*, **47**, 537 (1960)
(*2*) Fujita-Ikeda, S., *J. Biochem.*, **49**, 267 (1961)
(*3*) Isemura, T., and Fujita, S., *J. Biochem.*, **44**, 443, 797 (1957)
(*4*) Gralén, N., *Kolloid Z.*, **95**, 188 (1941)
(*5*) Gralén, N., Dissertation, Uppsala, (1944)
(*6*) Frith, E. M., *Trans. Faraday Soc.*, **41**, 90 (1945)
(*7*) Alfrey, T., Bartovics, A., and Mark, H., *J. Am. Chem. Soc.*, **64**, 1557 (1942)
(*8*) Schachman, H. K., "Ultracentrifugation in Biochemistry," Academic Press, New York and London, p. 151 (1959)
(*9*) Johnston, J. P., and Ogston, A. G., *Trans. Faraday Soc.*, **42**, 789 (1946)
(*10*) Schachman, H. K., and Kauzmann, W. J., *J. Phys. Colloid Chem.*, **53**, 150 (1949)
(*11*) Charlewood, P. A., *J. Am. Chem. Soc.*, **79**, 776 (1957)
(*12*) Scheraga, H. A., and Mandelkern, L., *J. Am. Chem. Soc.*, **75**, 179 (1953)
(*13*) Scheraga, H. A., Carrole, W. R., Nims, L. F., Sutton, E., Backus, J. K., and Saunders, J. M., *J. Polymer Sci.*, **14**, 427 (1954)
(*14*) Edelhoch, H., *J. Am. Chem. Soc.*, **79**, 6100 (1957)

(*15*) Doty, P., and Yang, J. T., *J. Am. Chem. Soc.*, **78**, 498 (1956); Yang, J. T., and Doty, P., *J. Am. Chem. Soc.*, **79**, 761 (1957)

(*16*) Fox, T. g., and Flory, P. J., *J. Phys. Colloid Chem.*, **53**, 197 (1949)

(*17*) Akabori, S., Ikenaka, T., Hanafusa, H., and Okada, Y., *J. Biochem.*, **41**, 803 (1954); Kuratomi, K., Ohno, K., and Akabori, S., *J. Biochem.*, **44**, 183 (1957)

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Guanidino Compounds from a Sea-Anemone, *Anthopleura japonica* Verrill

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In the course of studies on the extractable naturally occurring guanidino compounds, paper chromatographic analyses of an extract from a sea-anenone by means of the Sakaguchi reaction showed the presence of a number of guanidino compounds. Chromatography and electrophoresis on paper with an eluate obtained from an ion-exchange resin column, on which above guanidino compounds were adsorbed, separated the compounds into nine members identified as taurocyamine, glycocyamine, γ-guanidino-β-hydroxybutyric acid, β-guanidinopropionic acid, γ-guanidinobutyric acid, γ-hydroxyarginine, arginine, agmatine and hydroxyagmatine (a new compound). Moreover, most of them were isolated as crystals from a large amount of the material.

The present paper deals with the separation, identification, isolation and characterization of these guanidino compounds.

EXPERIMENTAL

Material

The sea-anemones (*Anthophopleura japonica* Verrill) were collected at the tidal zone of the Myoken Island in Hakata Bay.

Methods

Preparation of Sea-Anemone Extracts—The washed materials were homogenized with water in a mixer. The homogenate was mixed with about 1.2 times its volume of ethanol and the suspension was allowed to stand overnight, followed by filtration through a cotton filter.

Paper Chromatography—A portion of a solution to be tested was subjected to one dimensional ascending technique on Toyo No. 52 filter paper cylinder, 40 cm. tall and 15 to 40 cm. wide, by using *n*-butanol-acetic acid-pyridine-water (4 : 1 : 1 : 2, by volume), *n*-butanol-acetic acid-water (4 : 1 : 2, by volume) and pyridine-isoamylacohol-acetic acid-water (8 : 4 : 1 : 4, by volume) as solvents. After development, the paper was allowed to dry at room temperature and stained with the Sakaguchi reagent (*1*).

Paper Electrophoresis—The procedure was carried out on Toyo No. 52 filter paper sheet, 15 cm. wide and 36 cm. long (the starting line was 8 cm. towards anode from the central line), with *M*/50 acetate buffer of pH 4.1 as the electrolyte solution and 400 volts for 2.5 hours in a horizontal position. The paper dried in an oven at 90°C was subsequently sprayed with the Sakaguchi reagent.

Fractionation of Amberlite IR-120—Ion exchange chromatographic procedures were performed always at room temperature. A column (2×100 or 5×100 cm.) of Amberlite IR-120 (CG-120), 100 to 200 mesh, in the acid form was first used to chromatograph the guanidino compounds in the extract. After washing the column with water, the compounds were eluted with an acid solution, 10 ml. or 30 ml. fractions being collected at the rate of 30 to 45 minutes per fraction. This procedure in a small scale (2×100 cm. column, 10 ml. fraction) was employed for quantitative determination of the guanidino compounds.

Fractionation on Dowex 1—Fractions from Amberlite IR-120 including the required guanidino compound were combined and concentrated. The concentrated fraction was passed through a column (2×60 cm.) of Dowex 1-X8, 200 to 400 mesh, in the hydroxyl form to separate the compound from non-guanidino and guanidino compounds responsible for contamination. The column used was previously equilibrated with CO_2-free alkali and washed free from excess alkali with CO_2-free distilled water. The elution of the guanidino compounds was performed with CO_2-free distilled water, and 10 ml. fractions were collected.

Chromatography on Dowex 50—The separation of glycocyamine from γ-guanidino-β-hydroxybutyric acid was accomplished by ion exchange chromatography on a column (2×100 cm.) of Dowex 50-X4, 200 to

400 mesh, in the acid form. Elution was carried out with 0.5 *N* HCl and the eluate was collected in 10 ml. fractions.

Determination of Guanidino Compounds—Weber's method (2) which modified Sakaguchi's reaction was employed for estimation of the guanidino compounds. By using 1 to 2 ml. of the fractions obtained with each fractionation on all ion exchange columns, the compounds were roughly estimated. For quantitative estimation, aliquots of the fractions obtained with fractionation on only Amberlite IR-120 (small scale) were used. A solution to be examined was diluted until the arginine equivalent became 1 to 12 μg./ml. Five ml. of the resulting solution was taken into a test tube, and, after addition of 1 ml. of a sodium hydroxide solution (according to the concentration of hydrochloric acid used for elution of guanidino compound on a column of Amberlite IR-120, see Results II, 10 to 40 per cent was used so that the final concentration became about 2 per cent) and 1 ml. of 0.02% α-naphthol solution, it was shaken in cold water. A required quantity of 1% sodium hypobromite solution was then added to develop a maximum coloration. Immediately after shaking, 1 ml. of 40% urea solution was added to decompose excess sodium hypobromite. The well mixed solution was analyzed with electric photometer at 500 mμ wave length. The amount of guanidino compound was estimated as arginine equivalent from standard curve of arginine.

Separation Procedure

An extract prepared from 45 g. of the materials was evaporated to dryness under reduced pressure and the residue dissolved in 10 ml. of water was poured onto a column (2×100 cm.) of Amberlite IR-120. After washing with water, the column was developed with 0.5 *M* acetic acid and then eluted with hydrochloric acid of stepwise increasing concentration from 1 to 4 *N*. All effluents and eluates were collected in 10 ml. fractions. An aliquot from each fraction was analyzed by the modified Sakaguchi reaction photometrically. The remaining aliquots of the fractions were combined peak by peak and evaporated separately *in vacuo*. Each residue was dissolved in a small amount of water and a portion of each solution was subjected to paper chromatography and paper electrophoresis.

Isolation Procedure

Isolation of the guanidino compound was performed from a large amount of the materials by means of ion-exchange resin column in the similar manner to the chromatographic separation described above. About 16 liters of the extract prepared from 1.2 kg. of the materials were passed through a column of Amberlite IR-120 (5×30 cm.), 50 to 100 mesh, to adsorb the guanidino compounds excepting taurocyamine. The column was washed with water until the washings were negative for the Sakaguchi reaction. Taurocyamine present in the effluent and washings from the column was not isolated on account of its small quantity. The contents of the column were poured into 2 liters of water and stirred. The supernatant together with the floating colloidal matter was discarded. After washing twice with water, the resin was set again as the same column as before. The column thus obtained was eluted with 5 liters of 4 *N* HCl. The resulting eluate was evaporated to dryness *in vacuo* and the evaporation was repeated once more after addition of 100 ml. of water. The residue was dissolved in 500 ml. of 50% ethanol, and the resulting solution freed from insoluble matter was concentrated to 50 ml. The concentrated solution was passed again through another column of Amberlite IR-120 (5× 100 cm.). After washing with 500 ml. of water, the column was eluted with hydrochloric acid of stepwise increasing concentration. Elution was carried out as follows: from tube 1 to 182, with 1.0 *N* HCl; from tube 183 to 330, with 1.5 *N* HCl; from tube 331 to 540, with 2 *N* HCl; from tube 541 to 774, with 3 *N* HCl; and above tube 775, with 4 *N* HCl, 30 ml. fractions being collected. An aliquot (about 2 ml.) of each fraction was analyzed semiquantitatively with the Sakaguchi color reaction. Six groups of the fractions corresponding to the peaks B, C, D, E, F and G found in the elution diagram (Fig. 2) were also obtained in this case. Isolation of the guanidino compounds from these fractions was performed as follows.

Isolation of Glycocyamine and γ-Guanidino-β-hydroxybutyric Acid—The fractions (tubes 265 to 314) containing glycocyamine and γ-guanidino-β-hydroxybutyric acid were contaminated considerably with such guanidino compounds as seen in Results III and with ninhydrin positive substances. The latter contaminates were easily removed from the combined and concentrated fraction above mentioned by further fractionation on a column of Dowex 1 (2×60 cm.). Although some of the Sakaguchi positive peaks other than glycocyamine and γ-guanidino-β-hydroxybutyric acid appeared in this fractionation, their amounts were too little to be identified. Tubes 102 to 136 contained above described two guanidino compounds. However, their quantities were not enough to be isolated as crystals, so that two batches obtained by the same treatment from 2.4 kg. of the materials were combined together. The combined solution was concentrated.

and then chromatographed on a column of Dowex 50 as described previously.

Glycocyamine was found in tubes 170 to 193 obtained from the Dowex 50 column. These farctions were combined and evaporated to dryness under reduced pressure. The residue dissolved in 5 ml. of water was passed again through another column of Dowex 1 for fractionation. As the fractions (tubes 35 to 38) thus obtained contained glycocyamine only, they were combined and evaporated to a small volume. On addition of ethanol to it, glycocyamine crystallized out. The yield was 6.5 mg. The crystals melted at 255–260°C with decomposition.

$C_3H_7O_2N_3$ Calcd. C 30.77, H 6.03, N 35.88
Found C 31.05, H 5.94, N 35.66

On the other hand, γ-guanidino-β-hydroxybutyric acid was found in tubes 199 to 234 obtained from the Dowex 50 column. The fractions were treated again with Dowex 1 as in the case of glycocyamine. From the combined and concentrated fractions (tubes 34 to 37), on addition of ethanol, γ-guanidino-β-hydroxybutyric acid was obtained as crystal. The yield was 2.5 mg., m.p. 237°C.

$C_5H_{11}O_3N_3$ Calcd. N 26.08
Found N 25.85

Isolation of β-Guanidinopropionic Acid—The fractions (tubes 369 to 390) contained β-guanidinopropionic acid contaminated with a considerable amount of ninhydrin positive substances. When the fractions were combined and then fractionated further on a column of Dowex 1, β-guanidinopropionic acid appeared in tubes 21 to 24. These fractions were combined and concentrated to a small volume *in vacuo*. Upon addition of ethanol to it, β-guanidinopropionic acid crystallized. It was recrystallized from aqueous ethanol. The yield was 18 mg., m.p. 209–211°C.

$C_4H_9O_2N_3$ Calcd. C 36.64, H 6.92, N 32.05
Found C 36.71, H 6.86, N 32.15

Isolation of γ-Guanidinobutyric Acid—From the fractions (tubes 480 to 515) γ-guanidinobutyric acid was isolated in the similar manner to the case of β-guanidinopropionic acid. After fractionasion on a column of Dowex 1, the contents of tubes 11 to 13 were concentrated and treated with ethanol. The crystals obtained were recrystallized from aqueous ethanol. The yield was 16 mg., m.p. 240–245°C.

$C_5H_{11}O_2N_3$ Calcd. C 41.37, H 7.64, N 28.95
Found C 41.22, H 7.37, N 29.13

Isolation of γ-Hydroxyarginine—The fractions (tubes 634 to 673) contained γ-hydroxyarginine contaminated with a small amount of ninhydrin positive-Sakaguchi negative substances. When the fractions were combined and then fractionated further on a column of Dowex 1, γ-hydroxyarginine appeared in tubes 11 to 13. These fractions were combined and concentrated to about 10 ml. *in vacuo*. By the addition of a sufficient amount of flavianic acid to the resulting solution which was acidified with sulfuric acid, the flavianate was obtained. After recrystallization twice from water, the crystals showed not only distinct melting point but also definite analytical results. Therefore, the flavianate was converted into hydrochloride as follows.

Two batches of the flavianate obtained by the same treatment from 2.4 kg. of the materials were combined together and converted into free base solution in the usual manner. The solution thus obtained was concentrated to 5 ml. under reduced pressure and fractionated again on another column (2×80 cm.) of Dowex 1 for further purification. Elution was carried out with CO_2-free water and the eluate was collected in 10 ml. fractions. The fractions (tubes 26 to 52), which gave a single spot for the Sakaguchi or ninhydrin reagent on paper chromatograms, were combined and concentrated to a small volume *in vacuo*. After being brought to about pH 6.5 with dilute hydrochloric acid, the solution was evaporated to a syrup. The syrup was dissolved in hot aqueous ethanol (1 : 1), and then absolute ethanol was addep to it until the solution became opalescent. When the solution was kept several days in a refrigerator, γ-hydroxyarginine monohydrochloride crystallized. It was recrystallized by dissolving into a minute amount of 70% hot ethanol and then adding absolute ethanol. The yield was 50 mg. It decomposed at 190–191°C and showed a rotation of $[\alpha]_D^{13}$+6.3° (2 per cent in 5 *N* HCl). The analytical data agreed well with those of γ-hydroxy-L-arginine monohydrochloride (*3*).

$C_6H_{14}O_3N_4 \cdot HCl$ Calcd. C 31.79, H 6.67, N 24.72
Found C 31.88, H 6.84, N 24.53

Isolation of Arginine and Hydroxyagmatine—The fractions (tubes 720 to 760) contained arginine and hydroxyagmatine contaminated with a small amount of ninhydrin positive-Sakaguchi negative substances. When a solution obtained by combining above fractions was fractionated further on a column of Dowex 1, hydroxyagmatine appeared in tubes 3 to 6, while arginine was found in tubes 8 to 12. Two batches of these fractions obtained from 2.4 kg. of the materials by the same treatment were combined in each compound, and crystallization was performed as follows.

The fractions containing arginine were combined and concentrated to crystallize as flavianate in the

usual manner. After recrystallization by dissolving into ammonia and then adding hydrochloric acid, the crystals decomposed at 256–258°C. The yield was 25 mg.

$C_6H_{14}O_2N_4 \cdot C_{10}H_6O_8N_2S$

Calcd. C 39.34, H 4.13, N 17.21
Found C 39.02, H 4.40, N 16.96

Much efforts were made without success to crystallize hydroxyagmatine from the fractions containing this compound.

Isolation of Agmatine—From the fractions (tubes 858 to 887), agmatine was isolated in the similar way to the case of β-guanidinopropionic acid. The fractions were combined and subjected to further fractionation on a column of Dowex 1. Tubes 3 to 6 obtained by this treatment were moderately concentrated and acidified to about pH 5.0 with sulfuric acid. The resulting solution was evaporated to a small volume *in vacuo*. Upon addition of methanol to it, agmatine sulfate crystallized. It was recrystallized from aqueous methanol. The yield was 50 mg., m.p. 229°C.

$C_5H_{14}N_4 \cdot H_2SO_4$ Calcd. C 26.31, H 7.06, N 24.55
Found C 26.14, H 6.96, N 24.79

RESULTS

I. Preliminary Experiment of the Presence of Guanidino Compounds in the Material

It was found by a two-dimensional chromatogram on paper that an extract from the material contained a number of the Sakaguchi-positive

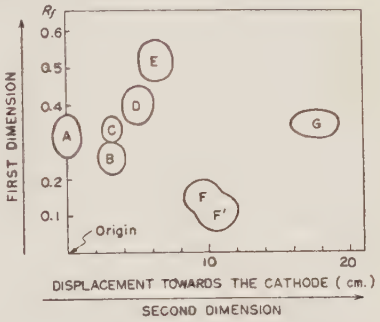

Fig. 1. Two-dimensional chromatogram of an extract from the material. The chromatogram was obtained by a technique involving ordinary chromatography on paper in the first dimensionw ith *n*-butanol-acetic acid-pyridine-water as a solvent, followed by electrophoresis in the second dimension with *M*/50 acetate buffer (pH 4.1) as an electrolyte solution. The Sakaguchi reagent was used for staining. Spots D, E and G are β-guanidinopropionic acid, γ-guanidinobutyric acid and agmatine, respectively. Spot A is an acidic, spots B, C, D and E are neutral and spots F, F′ and G are basic guanidino compounds.

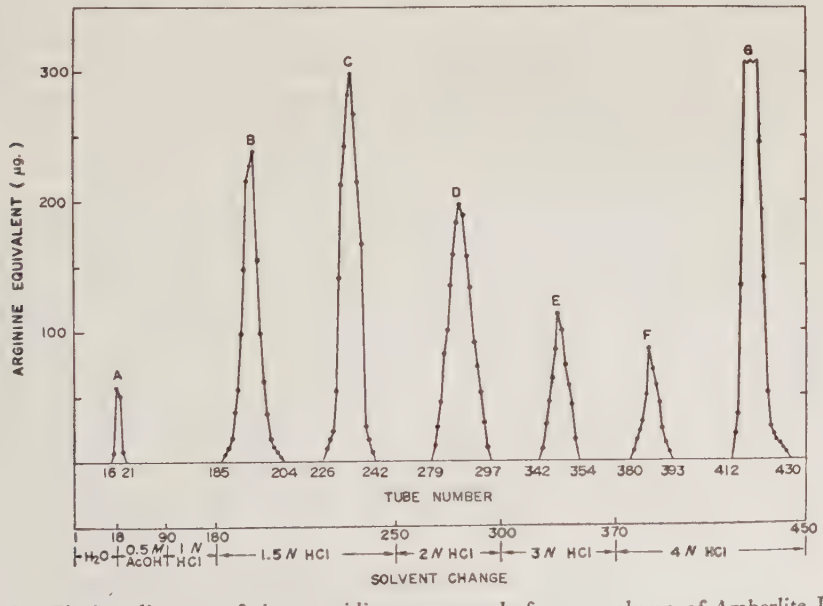

Fig. 2. Elution diagram of the guanidino compounds from a column of Amberlite IR-120.
Resin: Amberlite CG-120 (100 to 200 mesh)·
Column: 2×100 cm. Fraction: 10 ml./fraction.
Load: Aqueous solution (10 ml.) of concentrated extract prepared from 45 g. of material.

compounds as shown in Fig. 1. Seven to eight spots were detected on the chromatogram, but clear indentifications of them except three spots of agmatine, β-guanidinopropionic acid and γ-guanidinobutyric acid were difficult by judging only from their positions on the chromatogram.

II. Separation and Estimation of Guanidino Compound—An extract prepared from 45 g. of the materials was chromatographed on a column (2×100 cm.) of Amberlite IR-120. The elution diagram of the guanidino compounds is shown in Fig. 2.

The chromatographic fractionation showed that the guanidino compounds contained in the material were separated clearly into seven peaks A, B, C, D, E, F and G. The amounts of total arginine equivalent of each peak were given in Table I. The results obtained

TABLE I

Total Arginine Equivalent of the Peaks Obtained from Chromatography on a Column of Amberlite IR-120

Peak number	A	B	C	D	E	F	G
Total arginine equivalent (μg.)	131	1374	2025	1721	638	447	3978

under the assumption that the guanidino compounds have the same color intensity as arginine, do not give, of course, the real

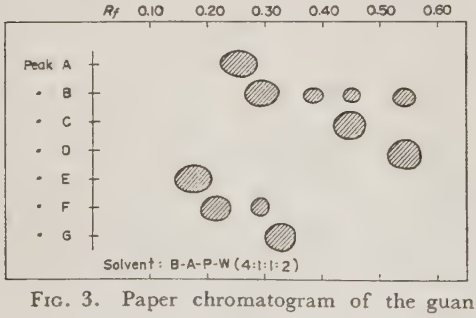

FIG. 3. Paper chromatogram of the guanidino compounds contained in each peak. The Sakaguchi reagent was used for staining. B-A-P-W: *n*-Butanol-acetic acid-pyridine-water.

quantities, since, for example, such guanidino compound which contains a hydroxyl group in the molecule as γ-hydroxyarginine was about 1/5 as active as arginine on the susceptibility to the Sakaguchi reaction. Accordingly, the data obtained here represent relative amounts of the guanidino compounds calculated as arginine.

III. Detection of Guanidino Compound in the Chromatographic Peaks—As shown in Fig. 3 and 4, it was found that each of the peaks A, C, D, E and G consisted of almost only one Sakaguchi-positive compound, while there were more than two compounds in the peaks B and F. *Rf* values of these spots and known guanidino compounds related to them are summarized in Table II.

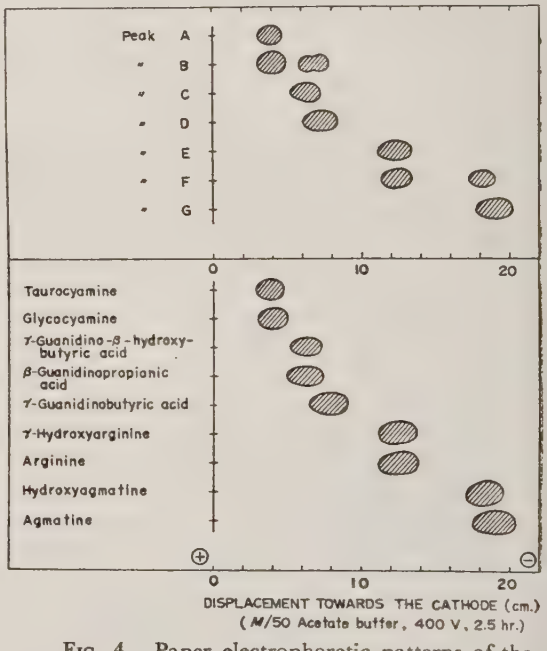

FIG. 4. Paper electrophoretic patterns of the guanidino compounds contained in each peak and of known compounds. The Sakaguchi reagent was used for staining.

The chromatographic and electrophoretic results showed that the peak A consisted of taurocyamine, the peak B of taurocyamine, glycocyamine, γ-guanidino-β-hydroxybutyric acid, β-guanidinopropionic acid and γ-guanidinobutyric acid, the peak C of β-guanidinopropionic acid, the peak D of γ-guanidinobutyric acid, the peak E of γ-hydroxyarginine, the peak F of arginine and an unknown compound, and the peak G of agmatine,

TABLE II

Rf Values of Unidentified Guanidino Compounds Contained in Each Peak and their Related Known Compounds

Sample	*Rf* Value		
	n-Butanol-acetic acid-pyridine-water	*n*-Butanol-acetic acid-water	Pyridine-isoamylacohol-acetic acid-water
Peak A	0.26	0.22	0.33
Taurocyamine	0.27	0.23	0.35
Peak B	0.29	0.22	0.24
	0.38	0.32	0.32
	0.45	0.44	0.38
	0.54	0.56	0.48
Glycocyamine	0.29	0.33	0.26
γ-Guanidino-β-hydroxy-butyric acid	0.38	0.43	0.36
Peak C	0.44	0.46	0.39
β-Guanidinopropionic acid	0.45	0.50	0.37
Peak D	0.54	0.57	0.49
γ-Guanidinobutyric acid	0.54	0.58	0.48
Peak E	0.17	0.19	0.15
γ-Hydroxyarginine	0.17	0.21	0.15
Peak F	0.21	0.24	0.17
	0.29		0.25
Arginine	0.21	0.25	0.18
Hydroxyagmatine	0.28	0.26	0.25
Peak G	0.33	0.27	0.30
Agmatine	0.34	0.29	0.29

respectively. Although the reason why taurocyamine, β-guanidinopropionic and γ-guanidinobutyric acids appeared in two peaks can not be explained satisfactorily, imperfect separation is conceivable. An unknown compound found in the peak F together with arginine was identified as hydroxyagmatine by comparing the paper chromatographic and paper electrophoretic behaviors of the compound with those of the decarboxylated product from γ-hydroxyarginine by the action of arginine decarboxylase (*4*).

IV. Isolation of the Guanidino Compounds—Isolation of the guanidino compounds was performed from a large amount of materials by means of a series of fractionation on ion-exchange resin columns as described above.

Taurocyamine which is not adsorbed on the column of Amberlite IR-120 was not isolated on account of its small quantity.

Glycocyamine, γ-guanidino-β-hydroxybutyric acid, β-guanidinopropionic acid, γ-guanidinobutyric acid, γ-hydroxyarginine, arginine and agmatine were isolated in pure forms from the fractions containing each compound. The analytical data of these compounds were well compatible with those of the reference compounds.

Although hydroxyagmatine could not be isolated as crystal, it was found as a feature, that this compound gives a characteristic Sakaguchi's reaction, the color of which is bright pink fading gradually to orange as equally seen in such guanidino compounds possessing a hydroxyl group in the molecule as γ-hydroxyarginine and γ-guanidino-β-hydroxybutyric acid.

DISCUSSION

Attempts to elucidate the biochemical characteristics of the naturally occurring guanidino compounds and their metabolism have been continued by many investigators. Recently the distribution of the guanidino compounds in nature has been investigated extensively and several new compounds have been found. Although considerable attentions have been paid to marine invertebrates, comparatively little is known of *Coelenterata*, one of the classes of marine invertebrates.

It was found that the procedures of chromatography on ion-exchange resin column were also a useful tool for the separation of guanidino compounds from an extract of material which contains a numbr of these componds, although estimation of guanidino compounds has been achieved for the most part by paper chromatographic techniques. However, there was a weak point in this method, *i.e.* some labile compound expected to occur could not be detected after treatment with acid. Accordingly, there is a place left for improvement.

The existence of taurocyamine in *Coelenterata*, which is known to be present predominantly in *Annelida* (*5*, *6*) was demonstrated here. Glycocyamine was found to be distributed rather widely in invertebrate (*5*, *6*, *7*). The occurrence of β-guanidinopropionic acid as a constituent of the living material was first proved in this study. It is well known that this compound is produced from arginine by chemical oxidation (*8*), although it has not yet been recognized as a naturally occurring product. Therefore, it is of interest to clarify the formation of this compound in the organism. γ-Guanidinobutyric acid was shown to arise in marine invertebrates by the oxidation of arginine through the action of L-amino acid oxidase (*7*) and to be widely distributed in *Mollusca*, *Echinodermata* and *Arthropoda* together with δ-guanidino-α-ketovaleric acid (*7*, *9*). It is interesting that γ-hydroxyarginine, which was found recently in a sea-cucumber (*3*), was also appeared in this sea-anemone, while the presence of arginine in almost all invertebrates is well known. The occurrence of agmatine in *Coelenterata*, which was found in some members of *Arthropoda* and *Mollusca* (*6*, *7*, *9*), was also confirmed here. The presence of arcaine in this sea-anemone reported in the previous study (*6*) was not demonstrated in this study. It seems doubtful, therefore, that arcaine is present in this organism.

In view of the existence of a considerable amount of γ-guanidinobutyric acid and agmatine in comparison with a small amount of arginine, it may be concluded that the two general catabolisms, oxidative deamination and decarboxylation reactions, came into operation simultaneously in this organism. It seems possible that γ-hydroxyarginine which is an analogue of arginine was also metabolized by the enzymes responsible for arginine. The evidences to support this assumption were already obtained. It was found that γ-hydroxyarginine was catabolized, though somewhat slowly than arginine, by the actions of arginase from liver (*3*) and arginine decarboxylase from *E. coli* (*4*). It was also proved that the compound was oxidized to corresponding keto acid by the action of L-amino acid oxidase from mussel and then to γ-guanidino-β-hydroxybutyric acid therefrom with hydrogen peroxide (*3*). γ-Guanidino-β-hydroxybutyric acid was actually found in this organism, and hydroxyagmatine was also detected by paper chromatography, although the latter compound could not be isolated as crystal. It follows from this that the above two guanidino compounds are also produced in this organism from γ-hydroxyarginine through the same metabolic pathways described above. It seems reasonable that the content of γ-hydroxyarginine was largely over than that of its metabolites in contrast with the relations between arginine and its metabolites, since the enzymes responsible for arginine were less effective on hydroxyarginine.

An attempt to isolate the acidic guanidino compound which was found on the two-dimensional chromatogram in the preliminary experiment was not successful. On the other

hand, hardly detectable guanidino compounds and the Sakaguchi negative ones in this material remained unidentified.

SUNNARY

The Sakaguchi positive nine guanidino compounds are detected from an extract of the sea-anemone, *Anthopleura japonica* Verrill, by means of chromatography and electrophoresis on paper. These compounds were identified as taurocyamine, glycocyamine, γ-guanidino-β-hydroxybutyric acid, β-guanidinopropionic acid, γ-guanidinobutyric acid, γ-hydroxy-L-arginine, arginine, agmatine and hydroxyagmatine, a new compound. Seven guanidino compounds of them excepting taurocyamine and hydroxyagmatine were isolated in pure form by a series of fractionation on ion-exchange resin columns and characterized.

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REFERENCES

(1) Makisumi, S., *J. Chem. Soc. Japan (Pure Chem. Sec.)*, **73**, 737 (1952)
(2) Weber, C. J., *J. Biol. Chem.*, **86**, 217 (1930)
(3) Fujita, Y., *Bull. Chem. Soc. Japan*, **32**, 439 (1959)
(4) Makisumi, S., *J. Biochim.*, **49**, 292 (1961)
(5) Thoai, N. V., Roche, J., Robin, Y., and Thiem, N. V., *Biochim. et Biophys. Acta*, **11**, 593 (1953)
(6) Sasaki, H., Fujita, Y., Makisumi, S., and Shibuya, S., *J. Japan. Biochem. Soc.*, **30**, 642 (1958)
(7) Thoai, N. V., Roche, J., and Robin, Y., *Compt. rend.*, **235**, 832 (1952); *Biochim. et Biophys. Acta*, **11**, 403 (1953)
(8) Mourgue, M., and Dokhan, R., *Acad. Des Sci.*, **29**, 1518 (1954)
(9) Fujita, Y., Makisumi, S., and Shibuya, S., *J. Japan. Biochem. Soc.*, **27**, 570 (1955)

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Decarboxylation of Hydroxyarginine and Canavanine by *Escherichia coli*

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It has been known that a strain of *Escherichia coli* decarboxylates L-arginine to agmatine and serves for estimation of L-arginine (*1*). γ-Hydroxyarginine, a structural analogue of arginine, discovered first in a sea-cucumber, *Polycheria rufescens* (*2*) was also found in a sea-anemone, *Anthopleura japonica* Verrill (*3*). The biological role of this compound is, if any, unknown. The compound is known to participate in two enzymatic reactions, as follows: (a) Hydrolysis by arginase to γ-hydroxyornithine and urea (*2*), and (b) oxidation by L-amino acid oxidase presumably through the corresponding keto acid to γ-guanidino-β-hydroxybutyric acid (*2, 3*). In the previous study (*3*) the present author found that a considerable amount of γ-hydroxyarginine exists in a sea-anemone together with a small amount of its decarboxylation product (hydroxyagmatine) and suggested that the latter would be formed by the action of arginine decarboxylase on the former in the organism. It has already been shown that canavanine, another structural analogue of arginine, is also decarboxylated to the corresponding amine by *Escherichia coli* (*4, 5*).

The present study was undertaken to demonstrate the decarboxylation of γ-hydroxyarginine by *Escherichia coli* and also to re-examine that of canavanine.

MATERIALS AND METHODS

Culture—The strains of *Escherichia coli* 7020 and No. 1 used in this study were kindly supplied by Prof. M. Suda of Osaka University. Cultivation was carried out according to the method of Gale (*1*). Acetone-ether dried powder of the bacteria suspended in *M*/5 phosphate-citrate buffer of pH 5.2 at the rate of 10 mg. dry weight per ml. was used for assay.

Substrates—L-Arginine monohydrochloride was a commercial product. γ-Hydroxy-L-arginine monohydrochloride was supplied by Dr. Y. Fujita of this Laboratory, who isolated the base from a sea-cucumber (*2*). L-Canavanine was an isolated product from jack been meal.

Assay—The decarboxylation was followed in the Warburg manometers and the estimation was carried out in air at 30°C. One ml. of the freshly prepared suspension containing 10 mg. of the *E. coli* powder was placed in the side arm of the manometer and a mixture consisting of 1.0 ml. of 0.01 *M* substrate solution and of 0.5 ml. of *M*/5 phosphate-citrate buffer (pH 5.2) in the main compartment. After equilibration, the content of the side arm was tipped into the main compartment and readings were taken until the gas evolution nearly ceased. Almost no consumption of oxygen was observed during the period of measurement of CO_2 evolution.

On the other hand, the decarboxylation was also followed by paper chromatography and paper electrophoresis. Ten ml. of the reaction mixture containing the substrate, the *E. coli* powder and the buffer in amounts similar to those described in the case of manometric method was shaken for 90 minutes at 30°C and then allowed to stand in a incubator with occasional shaking for 20 hours. In the course of incubation, an aliquot was taken out from time to time, heated in a boiling water-bath for 5 minutes and freed from the coagulated matter by centrifugation. The resulting solution was subjected to paper chromatography and paper electrophoresis.

Paper Chromatography and Paper Electrophoresis—The paper chromatography was carried out by using the one dimensional ascending method as described previously (*3*). The solvent used was *n*-butanol-acetic acid-pyridine-water (4 : 1 : 1 : 2, by volume). The paper electrophoresis was carried out on six strips of Toyo filter paper No. 52 (2×36 cm.) in *M*/30 phosphate buffer of pH 6.8 with 400 volts for 2 hours. The staining was performed by spraying with the Sakaguchi

or an alkaline nitroprusside-ferricyanide reagent (*6*), and a ninhydrin reagent.

Fractionation on Amberlite IR-120 and Dowex 1—The isolation of the decarboxylation product was performed from a reaction mixture with large scale by means of ion exchange chromatography on a column (2×40 cm.) of Amberlite IR-120 (CG-120), 100 to 200 mesh, followed by on a column (2×60 cm.) of Dowex 1-X8, 200 to 400 mesh. The operation was the same technique in principle as that described previously (*3*).

RESULTS

Decarboxylation of Arginine, γ-Hydroxyarginine and Canavanine by E. coli 7020 and No. 1—The results of the two sets of experiments by the manometric method with *E. coli* 7020 and with *E. coli* No. 1 are shown in Fig. 1, a and b.

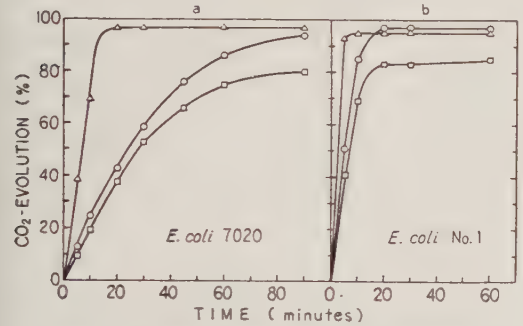

Fig. 1. Time course of decarboxylation of arginine, γ-hydroxyarginine and canavanine by *E. coli* 7020 and No. 1. Reaction mixture: 1 ml. of 0.01 *M* substrate, 0.5 ml. of the buffer, and 1 ml. of the cell suspension. Temperature, 30°C.
—△— Arginine. —○— γ-Hydroxyarginine.
—□— Canavanine.

The suspensions of these dried bacteria degraded arginine, γ-hydroxyarginine and canavanine, though there were some differences in the rates of decarboxylation as follows. In the case of *E. coli* 7020, the decarboxylation of arginine amounted to approximately 100 per cent after 20 minutes from starting, while those of γ-hydroxyarginine and canavanine were only about 40 per cent. On the other hand, after 90 minutes the decarboxylation of γ-hydroxyarginine reached to nearly 100 per cent, whereas that of canavanine stopped in about 80 per cent which was the maximum value of enzymatic decarboxylation for canavanine. These decarboxylation reactions proceeded substantially according to the first-order kinetics and the velocity constants for arginine, γ-hydroxyarginine and canavanine were in the ratio of approximately 1 : 1/4 : 1/5, respectively. In the case of *E. coli* No. 1, however, all the reactions proceeded more rapidly and gas evolutions almost ceased after 20 minutes, reaching to their end points. The reason why CO_2 evolution from canavanine by the action of dried cells of *E. coli* ceases at about 85 per cent is obscure.

Detection of the Decarboxylation Products—Fig. 2 and 3 show the results of time course

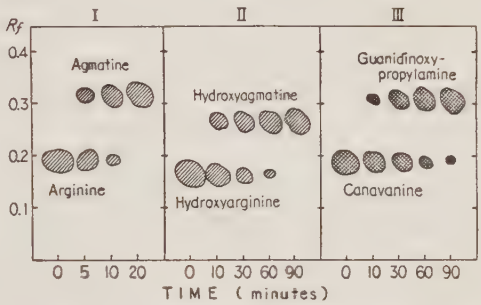

Fig. 2. Time-course chromatograms of the reaction mixtures during decarboxylation by *E. coli* 7020. Solvent: *n*-butanol-acetic acid-pyridine-water (4 : 1 : 1 : 2). Staining: The Sakaguchi reagent was used for I and II, and the alkaline nitroprusside-ferricyanide reagent for III, where I, II, and III represent to be Arginine, γ-Hydroxyarginine, and Canavanine, respectively.

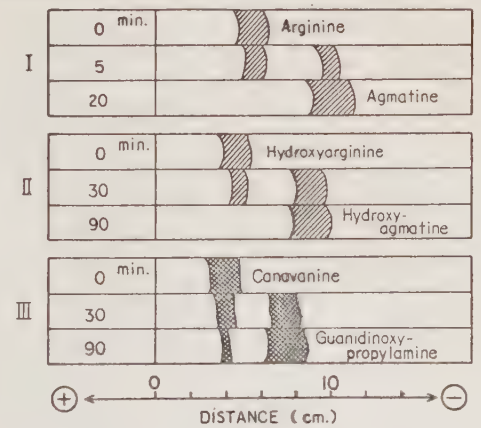

Fig. 3. Paper electrophoretic patterns of the reaction mixtures during decarboxylation by *E. coli* 7020. The staining reagents and the notations (I, II, and III) are the same as described in Fig. 2.

studies of the reaction with *E. coli* 7020 by paper chromatography and paper electrophoresis as several representative examples. As can be seen from them, the amounts of each substrate continued to decrease during incubation, while new compounds corresponding to amines continued to increase in amounts proportional to CO_2 evolution (Fig. 1). Even though prolonged incubation was carried out for 10 to 20 hours with *E. coli* 7020 or No. 1, no other spots than the amines were detected on the chromatograms.

The agmatine yielded from arginine was identified by paper chromatographic and electrophoretic comparisons with the authentic specimen. The new compound derived from γ-hydroxyarginine was assumed tentatively as hydroxyagmatine for following reasons: (a) it was formed from γ-hydroxyarginine by decarboxylation; (b) it gave both the Sakaguchi and ninhydrin reactions; and (c) its paper electrophoretic behavior was similar to that of agmatine. The compound formed from canavanine was identified by comparing the paper chromatographic and electrophoretic behaviors with those reported by Suzuki *et al.* (*4*) and Hagihara (*5*). It, as in canavanine, gave positive spot on paper with the alkaline nitroprusside-ferricyanide and the ninhydrin reagents, but not with the Sakaguchi reagent. The identification of the latter two decarboxylation products was confirmed by isolating them from the reaction mixtures as follows.

Isolation of the Decarboxylation Products

Hydroxyagmatine from γ-Hydroxyarginine—A reaction mixture (175 ml.) containing γ-hydroxyarginine, the dried cells of *E. coli* No. 1 and the buffer in amounts similar to those described in the case of manometric method was incubated at 30°C for an hour. The incubation mixture was freed from the cells by centrifugation, concentrated to about 20 ml. under reduced pressure, and passed through a column of Amberlite IR-120. The column was washed with 120 ml. of water, then eluted first with 1 *N* HCl, subsequently with 2 *N* HCl, and 20 ml. fractions were collected.

Trace amount of a Sakaguchi positive substance was eluted in each tube 29 to 32, however, these fractions were discarded. At tube 55 the elution with 2 *N* HCl started. Hydroxyagmatine was found in tubes 105 to 130. These fractions were combined and evaporated to dryness *in vacuo*, the evaporation being repeated once more after the addition of 20 ml. of water. The resulting syrup was dissolved in 5 ml. of water and the solution was passed through a column of Dowex 1. The column was then eluted with CO_2-free water and 10 ml. fractions were collected. Almost all hydroxyagmatine appeared in tubes 4 to 11. These fractions were united and concentrated to a small volume under reduced pressure. The general methods for crystallization from the solution as sulfate, hydrochloride, hydroperchlorate or nitrate were tested, but no crystal was obtained. Therefore, the compound was converted into picrate as follows.

When a sufficient amount of picric acid was added to the solution after slightly acidifing with sulfuric acid, the picrate precipitated rapidly from the solution. The picrate was recrystallized three times from water. After separation, the crystals were allowed to dry in air at room temperature. The yield was 230 mg. (45 per cent). It decomposed at 221°C with sintering. The analytical data of the product were well compatible with the composition assumed tentatively as hydroxyagmatine (δ-guanidino-γ-hydroxybutylamine) dipicrate.

$C_5H_{14}ON_4 \cdot 2(C_6H_3O_7N_3)$

Calcd. C 33.78, H 3.33, N 23.18
Found C 33.85, H 3.42, N 22.95

The free base from the picrate gave the specific coloration as in the case of γ-hydroxyarginine with the Sakaguchi reagent (*3*). Chromatographic *Rf* values with several different solvents and electrophoretic mobility of the compound were identical with those of the naturally occurring one described in the previous paper (*3*).

γ-Guanidinoxypropylamine from Canavanine—A reaction mixture (325 ml.) containing canavanine, the dried cells of *E. coli* No. 1 and the buffer in proportion similar to that described

in the case of manometric method was incubated at 30°C for 2 hours. The reaction mixture was then treated with the same manner as in the case of hydroxyagmatine. After fractionation on a column of Amberlite IR-120, the fractions (tubes 110 to 150) which gave only one spot with the ninhydrin or the alkaline nitroprusside-ferricyanide reagent on a paper chromatogram were combined and evaporated to syrup. When a solution dissolving the syrup in 10 ml. of water was fractionated on a column of Dowex 1, γ-guanidinoxypropylamine appeared in tubes 4 to 15. These fractions were combined and concentrated to a small volume. After being brought to about pH 5 with sulfuric acid, to the solution were added, drop by drop, about three times of its volume of methanol to complete the crystallization. The sulfate thus obtained was recrystallized from water-methanol mixture. The yield was 190mg. (64 per cent). It decomposed at 237°C. The analytical data showed the compound to be γ-guanidinoxypropylamine sulfate.

$C_4H_{12}ON \cdot H_2S_4O_4$ Calcd. C 20.87, H 6.13, N 24.34
Found C 20.91, H 6.17, N 24.08

DISCUSSION

It has been reported that although the decarboxylase is specific for the substrate, lysine decarboxylase attacks hydroxylysine slowly, and it has been concluded that substitution of hydroxyl group in a position other than in the three essential polar groups of the substrate molecule (the α-NH_2, the -COOH or the third terminal polar group) slows but does not prevent decarboxylation; consequently hydroxy-amino acids are attaked (*1*). It has been further reported that the strain of *E. coli* 7020 decarboxylates canavanine (*4*, *5*), though the organism is known to be specific for the decarboxylation of arginine alone (*7*).

The results of the present study clearly demonstrated that both γ-hydroxyarginine and canavanine were decarboxylated to the corresponding amines by the action of *E. coli* 7020, even though the rates of decarboxylation of the compounds were more slowly than that of arginine.

On the other hand, it has been known that the strain of *E. coli* No. 1 which possesses the ability to decarboxylate lysine, arginine, glutamic acid, histidine and ornithine, also decarboxylates canavanine (*5*). It is reasonable, therefore, that the strain of *E. coli* No. 1 attacked hydroxyarginine and canavanine more rapidly in comparison with *E. coli* 7020, since the latter organism has seemingly lower activity for decarboxylation of the amino acids than the former.

Basing on the results of experiments which showed the formation of neutral compounds in the course of prolonged incubation of arginine and canavanine with *E. coli* No. 1, Hagihara suggested previously that the decarboxylation products from them were metabolized further to the corresponding carboxylic acids (*5*). However, the formation of such neutral compounds found by Hagihara could not be confirmed in the present study. It is considered, therefore, that the oxidative degradation observed by him may not be the essential action of the dried cells of *E. coli*.

The present study has found the third enzymatic reaction of γ-hydroxyarginine. This finding supports the view that the enzymatic reactions in which arginine participates may be almost applicable to γ-hydroxyarginine and canavanine.

SUMMARY

Two strains of *Escherichia coli* 7020 and No. 1 were found to decarboxylate γ-hydroxyarginine and canavanine to form the corresponding amines.

The decarboxylation products from γ-hydroxyarginine and canavanine were isolated and identified as hydroxyagmatine (δ-guanidino-γ-hydroxybutylamine) and γ-guanidinoxypropylamine, respectively.

The author wishes to express his thanks to Prof. S. Shibuya for his valuable advice, and Assistant Prof. N. Izumiya for his interest. This study was supported in part by a Grant in Aid for Scientific Research from the Ministry of Education.

REFERENCES

(1) Gale, E. F., *Biochem. J.*, **39**, 52 (1945); **41**, vii (1947)
(2) Fujita, Y., *Bull. Chem. Soc. Japan*, **32**, 439 (1959)
(3) Makisumi, S., *J. Biochem.*, **49**, 284 (1961)
(4) Suzuki, T., Muraoka, S., and Konobu, K., *J. Pharm. Soc. Japan*, **74**, 540 (1954)
(5) Hagihara, F., *J. Pharm. Soc. Japan*, **76**, 613 (1956)
(6) Makisumi, S., *J. Chem. Soc. Japan* (*Pure Chem. Sec.*), **73**, 737 (1952)
(7) Gale, E. F., "Methods of Biochemical Analysis," Interscience Publishers, Inc. **IV**, 299 (1957)

The Journal of Biochemistry, Vol. 49, No. 4, 1961

Studies on Enzymatically Active Fragments of Pepsin

II. Isolation and Purification of Fragments with Peptic Activity

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Previous papers (*1*, *2*) have shown that enzymatically active fragments were produced by dialysis autolysis of a crude pepsin preparation most favorably at pH 4. By chromatographing the dialysate on a column of Amberlite IRC-50 (XE 64) the active fragments in the dialysate were separated at least into three components and almost a half of the activity was found in one of these components. The main component, however, was not homogeneous but a mixture of polypeptides with molecular weights smaller than 10,000. The purification of this component was carried out with a column of Dowex 50-X 2, with the result that approximately 70 per cent of the activity was eluted into a single fraction. Examination with continuous paper electrophoresis revealed that this fraction was still not homogeneous but could be divided into two components, a major, enzymatically active component and a minor inactive component. The further purification of the active polypeptide is now in progress.

MATERIALS AND METHODS

Pepsin—A commercial pepsin preparation from N.B.C., 1 : 10,000 U.S.P. was employed.

Determination of Proteolytic Activity (*3*)—A 0.6% Hammersten's casein solution of pH 1.8 was used as substrate. The procedure for estimation was the same as described previously (*1*, *2*). In this paper the protease activity is expressed as mg. tyrosine produced in the digestion mixture per mg. of nitrogen of the enzyme, and the specific activity is expressed as μg. value of tyrosine produced per mg. of nitrogen of the enzyme in a period of one minute and symbolized as $[PU]^{cas.\ 275,\ A.}_{\mu g.\ tyr.}$.

Estimation of Enzyme Concentration—The modified Folin method of Lowry *et al.* (*4*) was used. Nitrogen was determined with a micro-Kjeldahl procedure.

Continuous Paper Electrophoresis—A modification of the apparatus of Grassman and Hanning (*5*) was used.

Dialysis Autolysis—The autolysis was performed in the same manner as described previously (*1*).

Chromatography of Dialysate on a Column of Amberlite IRC-50 (XE 64)—The dialysate containing active fragments or a solution made by dissolving lyophilized dialysate in distilled water was chromatographed by passage through a column of Amberlite IRC-50 (XE 64) using *M*/10 acetate buffer of pH 4.0 and ionic strength 0.1 as eluent. The fraction containing the main active component in the eluate was referred to as fraction F-1.

Purification of Fraction F-1 by Chromatography with Dowex 50-X2—The component included in fraction F-1 was chromatographed on a column of Dowex 50 X2 (H-form) with distilled water.

Ultracentrifugation—The sedimentation patterns of these partially purified fragments were observed by use of a Spinco Ultracentrifuge Model E. For this purpose, these fractions were lyophilized and then dissolved in distilled water so that an approximately 1% enzyme concentration resulted. The apparent sedimentation constant was measured from the rate of sedimentation.

Estimation of pH—All pH values were determined with the glass electrode by means of a Horiba pH meter Model H.

RESULTS AND DISCUSSION

Chromotographic Separation of Peptic Active Fragments in Dialysate Using a Column of Amberlite IRC-50 (XE 64)—As mentioned in the previous paper (*2*), five components were obtained by subjecting the dialysate to column chromatography. Fig. 1 shows the fractionation diagram of the components yielded in the dialysate. Three of these fractions, F-1, F-2, and F-3 were responsible for the peptic

activity. It was found that approximately 50 per cent of the total peptic activity in the dialysate was in fraction F-1. The recoveries of nitrogen and peptic activity by this procedure were 75 and 85 per cent, respectively. Even when a concentrated solution of a lyophilized dialysate were used as a starting solution for chromatography in place of the outer solution of dialysis, almost the same fractionation diagram as above was obtained except that there were six instead of five peaks.

Ultracentrifugation of the F-1 Component—The sedimentation pattern of the F-1 component is illustrated in Fig. 2, indicating that it consists of small molecular substances. The fraction was, however, not homogeneous but seemed to be a mixture of polypeptides having different molecular weights. The sedimentation constant of a polypeptide which had the largest sedimentation rate in the mixture was estimated by Baldwin's method (*6*) and gave an *st* value of 0.71×10^{-13}. This value for *st* seems to indicate that its molecular weight is smaller than 10,000, assuming its molecular shape to be globular. There is little doubt, therefore, that at least one small polypeptide in fraction F-1, which is apparently produced by the degradation of a crude pepsin, possessed peptic activity.

Chromatographic Purification of Fraction F-1 Using a Column of Dowex 50-X 2 (H-form) with Distilled Water—Though a single peak was obtained by rechromatography of fraction F-1 on a column of Amberlite IRC-50 (XE 64), it was divided into four components by

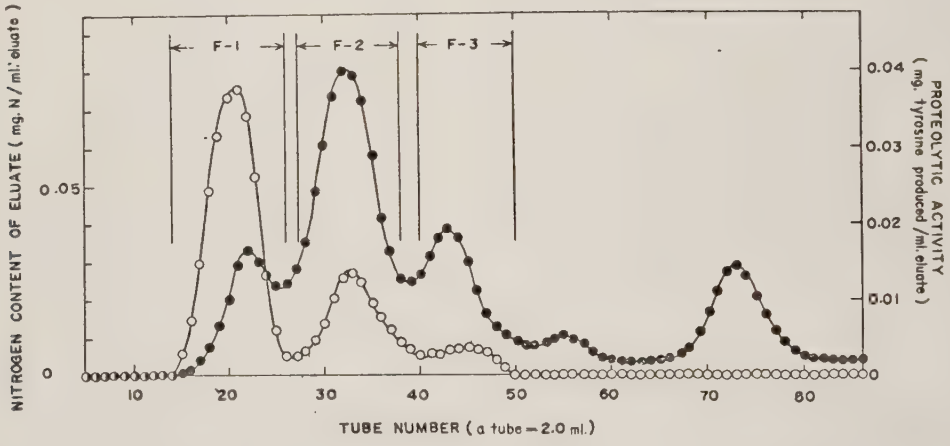

Fig. 1. Chromatogram of the dialysate produced by dialysis autolysis of crude pepsin on a column (30×1 cm.) of Amberlite IRC-50 (XE 64) with *M*/10 acetate buffer of pH 4.0 and ionic strength 0.1. Nitrogen content of eluate (●), mg. nitrogen/ml. eluate; Proteolyic activity (○), mg. tyrosine produced in digestion mixture/ml. eluate.

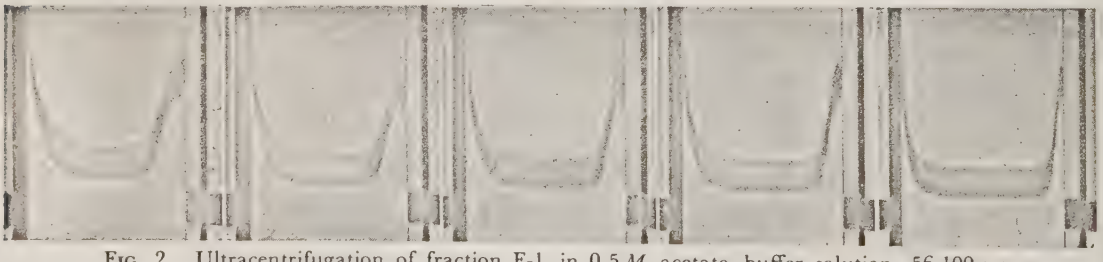

Fig. 2. Ultracentrifugation of fraction F-1 in 0.5 *M* acetate buffer solution, 56,100 r.p.m. The first exposure is at the extreme right. Intervals between exposure are 16, 8, 16, and 16 minutes, respectively.

subjecting it to column chromatography with Dowex 50-X 2, as shown in Fig. 3. Three of these components, F-1-a, F-1-b, and F-1-c, were found to have peptic activity. In this case, 95 per cent of the activity eluted, corresponding to approximately 70 per cent of the total peptic activity contained initially in fraction F-1, was found in fraction F-1-a. This fraction accounted for 14–20 per cent of the total nitrogen in fraction F-1. Thus the recoveries of nitrogen and peptic activity by this procedure were 85–90 and 75–80 per cent, respectively. When a buffered resin and *M*/10 acetate buffer of pH 4.0 were used in place of resin of H-form and distilled water, the total recoveries of nitrogen and peptic activity became 95 and 90 per cent respectively.

Ultracentrifugation of Fraction F-1-a—Fig. 4 shows the sedimentation pattern of the F-1-a component. The sedimentation constant of this fraction was estimated by Baldwin's method (*6*) and gave an *st* value of 0.56×10^{-13}, which was smaller than that of a polypeptide which had the largest sedimentation rate in fraction F-1. This value for *st* is an evidence that a small peptide produced by the degradation of a crude pepsin is responsible for peptic activity.

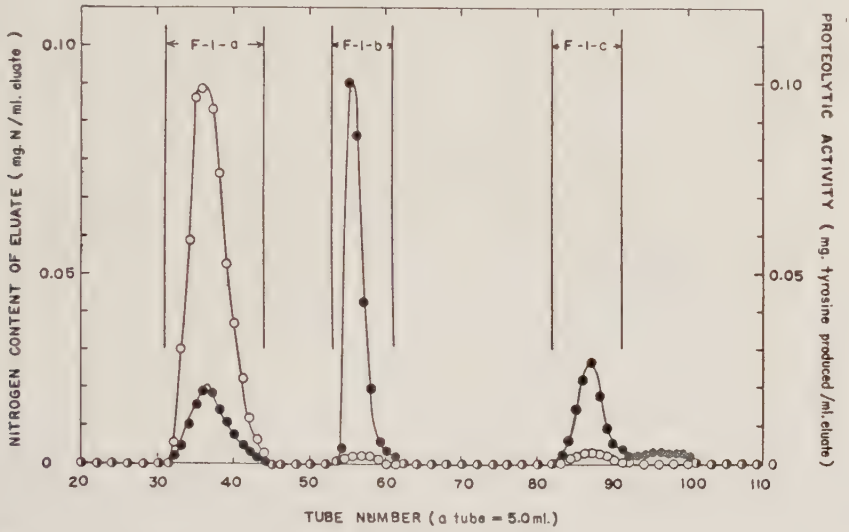

FIG. 3. Chromatogram of fraction F-1 on a column (110×2.2 cm.) of Dowex 50-X2 (H-form) with distilled water.

Nitrogen content of eluate (●), mg. nitrogen/ml. eluate; Proteolytic activity (○), mg. tyrosine produced in digestion mixture/ml. eluate.

FIG. 4. Sedimentation behavior of fraction F-1-a in the ultracentrifuge. The first picture (on the extreme right) was taken 30 minutes after 56,100 r.p.m. was reached; the subsequent photographs were taken at consecutive 16 minute intervals.

Nature of the Active Fragment in Fraction F-1-a

a) Heat Stability—After keeping one ml. aliquots of the pH 4.0 solution of fraction F-1-a for 30 minutes at various temperatures, *i.e.*, 40°C, 45°C, 50°C, 55°C, 60°C, 65°C, 70°C, 75°C, and 80°C, their activities were measured at pH 1.8 and 37°C. The results were represented in per cent of inactivation (Fig. 5) in contrast to the activity given without heating. As upon heating at pH 4.0 and 70°C for 30 minutes, 97.8 per cent of activity were lost, it can be said that the active fragment was not so heat stable as was stomach cathepsin (*7*), but has rather similar stability to native pepsin.

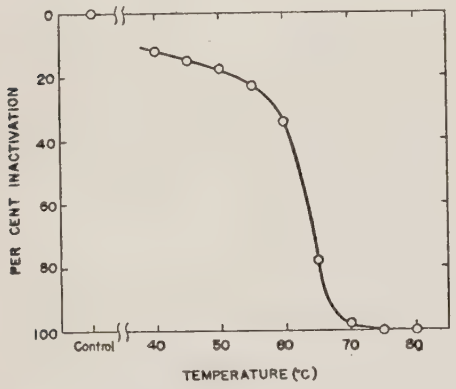

Fig. 5. Heat stability of fraction F-1-a. The activity was measured by the incubation for 10 minutes at pH 1.8 and 37°C after standing for 30 minutes at the indicated temperature at pH 4.0.

b) Proteolytic Activity as Function of pH—The pH-dependence of activity of fraction F-1-a was studied by measuring the proteolytic activities at pH range from 1.8 to 6.0 with 0.6% casein, and from 2.0 to 4.6 with 0.6% bovine hemoglobin. Fig. 6 shows the pH-activity curve of F-1-a and demonstrates that the range of optimal pH for the activity of the active fragment is wider than that of native pepsin.

Hemogeneity of the Active Fragment—Although the rechromatography of fraction F-1-a on a column of Dowex 50-X 2 gave a single symmetrical peak, the active fragment was further divided into two components by continuous paper electrophoresis using *M*/10 hydrochloric acid-potassium biphthalate buffer (Clark-Lubs buffer) of pH 3.2, at a constant gradient with a field strength of 9.1 volts per cm. at room temperature. A similar pat-

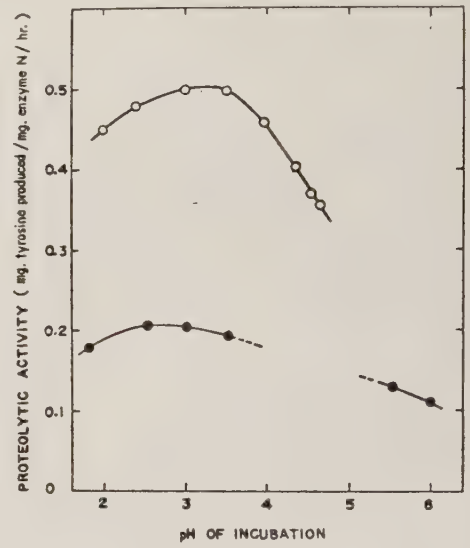

Fig. 6. pH-Activity curves of fraction F-1-a. The activity was measured by the incubation for one hour at 37°C with 0.6% casein (●), or 0.6% bovine hemoglobin (○).

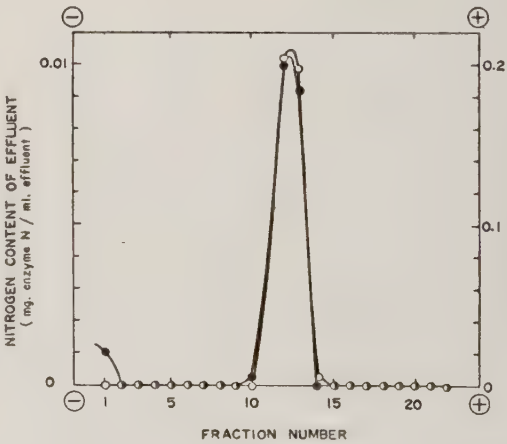

Fig. 7. Fractionation diagram of fraction F-1-a by continuous paper electrophoresis. Conditions: *M*/10 HCl-potassium biphthalate buffer of pH 3.2 and ionic strength 0.1, 200 volts/21 cm., 21 mA/23 cm., Tōyōroshi No. 50, 21 × 23 cm.

Nitrogen content of effluent (●), mg. nitrogen/ml. effluent; Proteolytic activity (○), mg. tyrosine produced in digestion mixture/ml. effluent/24 hrs.

tern was also obtained at pH 4.0 and field strength of 18.2 volts per cm., except that a major active component which flowed into fractions 12 and 13 at pH 3.2 as shown in Fig. 7, shifted to the fraction 19 at pH 4.0, and a minor inactive component which flowed into fraction 1 and the cathode at pH 3.2 in Fig. 7, shifted to fraction 7 at pH 4.0. The evidence apparently implies that fraction F-1-a did not consist of a single homogeneous component, so that further purification is still necessary.

Recoveries of Nitrogen and Proteolytic Activity of the Active Fragment—Table I shows the recoveries of nitrogen and proteolytic activity in the course of purification. Difficulties of

TABLE I

Recoveries of Total Nitrogen and Proteolytic Activity of the Active Fragment

Preparation	Recoveries of total nirogen[1] %	Recoveries of total proteolytic activity[1] %	$[PU]^{cas.,275,A}_{\mu g.\ tyr.}$
crude pepsin preparation			13,060
dialysate	33	1.2	450
fraction F-1	3.3	0.6	2,300
fractionF-1-a	0.6	0.4	8,300

1) Regarding total nitrogen and proteolytic activity of starting material of dialysis autolysis as 100 per cent.

the regulation of autolysis seem to cause the low yield of the active fragment of pepsin. However, approximately one third of the total proteolytic activity produced in the dialysate were obtained in fraction F-1-a. The specific activity of fraction F-1-a was approximately 18 times that of dialysate, and it corresponded to approximately one third of that of pepsin per unit nitrogen. While the fact, that the activity in the dialysate is maintained almost without loss through the two steps of chromatography, indicates that the active fragment is remarkably stable during the chromatographic procedure especially considering that such a strongly acidic resin as Dowex 50 was used, it was, on the other hand, easily ianctivated by the lyophilization.

However, the fact that such pan active fragment of small molecular weight can be obtained, leads to interesting speculation regarding the nature of the active center of enzyme. The removal of a large part of molecule would be expected to cause drastic changes in the configuration of remaining part of the molecule possibly affecting the three dimensional structure of the active center. The fragment obtained, however, was still enzymatically active. There are two possible explanations for the fact that such a fragment still has activity. One supposes that the active center is extremely stable, the stability depending upon the integrity of disulfide linkages, which are assumed to hold the active center together, preventing the transformation. Thus the rigid active center would retain its activity to a large extent. Another is concerned with the flexibility of the active center involving the induced fit type specificity proposed by Koshland (*8*). If there remains any ability of the flexible structure of the fragment to reform the active center in spite of the configurational change caused by the significant partial hydrolysis of the pepsin molecule, the active center would be induced to fit the substrate by mutual interaction between fragment and substrate. Thus the fragment could remain active. It is impossible, however, to decide which is more probable in this case.

SUMMARY

1. Threecomp onents having peptic activity were separated by column chromatography with Amberlite IRC-50 (XE 64) of the dialysable active fragments formed by dialysis autolysis of a crude pepsin preparation.

2. A component, F-1, containing almost a half of the activity of the eluate was revealed to be a mixture of small molecular polypeptides, of which each of the molecular weights was estimated to be smaller than 10,000 by measuring the sedimentation constant.

3. The F-1 component was further purified by column chromatography using Dowex

50-X 2, yielding fraction F-1-a, which involved approximately 95 per cent of the total proteolytic activity of the eluate.

4. The sedimentation constant of the F-1-a component was 0.56×10^{-13}.

5. Heat-stability and pH-activity curves of the F-1-a component were obtained. The range of optimal pH for the activity of the active fragment was wider than that of native pepsin.

6. The active fragment contained in the F-1-a component was further divided into two components, a major active component and a minor inactive component by continuous paper electrophoresis.

7. The specific activity of the partially purified active fragment (F-1-a) was approximately one third of that of pepsin.

We wish to express our thanks to Drs. M. Sonenberg and C. C. Bigelow, Sloan-Kettering Memorial Institute for Research in Cancer and Allied Diseases, for their help during preparation of the manuscript.

REFERENCES

(*1*) Funatsu, M., and Tokuyasu, K., *J. Biochem.*, **46**, 1441 (1959)
(*2*) Funatsu, M., and Tokuyasu, K., *Proc. Japan Acad.*, **35**, 139 (1959)
(*3*) Hagiwara, B., Yoneya, T., Yamamoto, T., and Komaki, T., In "*Kōso Kenkyuhō*" (ed. by Akabori, S.), Asakura-shoten, Tokyo, **Vol. 2**, p. 238 (1956)
(*4*) Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.*, **193**, 265 (1951)
(*5*) Grassmann, W., and Hanning, K., *Z. physiol. Chem.*, **292**, 32 (1953)
(*6*) Baldwin, R. L., *Biochem. J.*, **55**, 644 (1953)
(*7*) Buchs, S., *Enzymologia*, **13**, 208 (1949)
(*8*) Koshland, D. E. Jr., In "*Symposium on Enzyme Reaction Mechanisms*", Wister Inst. Anat. Biol., Philadelphia, P. 245 (1959)

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Glycogen Phosphorylase Activities of Tumors, Regenerating Rat Liver and Suckling Rat Liver

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Recently Nierenberg reported that the content of glycogen phosphorylase in various ascites tumors of mouse is much lower than that in normal mouse liver (*1*, *2*). The results presented here using ascites tumors and solid hepatoma confirmed his finding. It was also shown that the phosphorylase activity is low in non-malignant tissues, such as regenerating rat liver and suckling rat liver, which have a high rate of cell division.

MATERIALS AND METHODS

Tumor Cells—The tumor cells and strains of rats and mice used in the present work are listed in Table I. DAB-hepatoma was obtained from rats fed a diet containing 0.08% DAB* for 150 days. AH-130 solid hepatoma was obtained by injection of AH-130 ascites hepatoma into the portal vein or directly into the lobes of the liver. About 10 days after transplantation, the solid tumors produced in the liver were used. Tissue homogenates were prepared from them by the following procedure; a host rat was anaesthetized with ether and after laparotomy the liver was perfused by the portal vein with cold 0.2 *N* sodium fluoride. Then the tumor areas of the liver were carefully separated from the normal areas. To 1 g. of solid tumor, 2 ml. of 0.1 *N* sodium fluoride was added and the mixture was homogenized in an all-glass Potter-Elvejhem homogenizer in an ice water bath for 2.5 minutes. Homogenates of normal rat and mouse liver were obtained by the same method.

Table I

Tumors Used

Tumor	Type of Tumor	Host
DAB-hepatoma	Solid	Sprague-Dawley rats
AH-130-hepatoma	Solid	Sprague-Dawley rats
AH-130-hepatoma	Ascites	Sprague-Dawley rats
Ehrlich carcinoma	Ascites	ddo-mice
MH-129-D hepatoma	Ascites	C_3H-mice
HeLa cells	Tissue culture	—

Ascites tumor cells (AH-130 hepatoma, Ehrlich carcinoma and MH-129 D hepatoma) were harvested by laparotomy and collected by centrifugation at 300 r.p.m. for 3 minutes. They were washed with cold 0.95% sodium fluoride until the contaminating erythrocytes had been removed. HeLa cells** were washed with cold 0.95% sodium fluoride. Ascites tumor cells and HeLa cells were homogenized by the same method described above. For phosphorylase assay, homogenates were diluted with 0.05 *N* sodium fluoride.

Regenerating Liver—Sprague-Dawley rats of 150 to 200 g. body weight were partially hepatectomized by the method of Higgins and Anderson (*3*). A homogenate of regenerating liver was prepared by the same method after it had been perfused with cold 0.2 *N* sodium fluoride.

Suckling Rat Liver—New-born rats of Sprague-Dawley strain were decapitated and the livers were immediately removed, put into cold 0.2 *N* sodium fluoride and homogenized as mentioned above.

Phosphorylase Assay—Phosphorylase activity was measured by the method of Sutherland and Wosilait (*4*), but in order to eliminate the error caused by spontaneous decomposition of glucose-1-phosphate, liberated inorganic phosphate was determined by Takahashi's method (*5*). Thus the whole procedure was as follows: 0.25 ml. of an adequately diluted homogenate was put into a glass-stoppered test tube and the reaction was started by adding 0.5 ml. of substrate solution (*4*). After incubation at 37°C for a definite period, the reaction was stopped by adding 2 ml. of a cold solution of 1% ammonium molybdate in 0.75 *N* sulfuric acid. Then the tube was immediately transferred to an ice-water bath and 4 ml. of *iso-*

* The following abbreviations are used; DAB, *p*-N, N'-dimethylamino azobenzene; RNA, ribonucleic acid; DNA, desoxyribonucleic acid.

** HeLa cells were kindly given by Dr. Y. Aoki of this institute.

butyl alcohol was added. The mixture was shaken vigorously for about 30 seconds. After standing for several minutes, 2 ml. of the *iso*-butyl alcohol layer was transferred to a new tube and 1 ml. of ethyl alcohol (absolute) and 2 ml. of 0.5% ascorbic acid solution were added. After mixing thoroughly, the tube was incubated for 20 minutes at 37°C. Its optical density was measured spectrophotometrically at 660 mμ. In each experiment, a time curve was constructed and Fig. 1 shows an example obtained with normal rat liver. Phosphorylase activity was expressed by μmoles of inorganic phosphate liberated per 10 minutes per mg. of total nitrogen of the homogenate used.

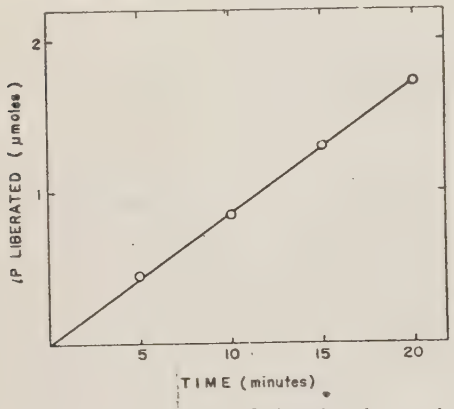

FIG. 1. Time curve of phosphorylase activity in a homogenate of normal rat liver. Phosphorylase activity was assayed as described in the text. The homogenate prepared as described in the text was diluted 40 fold with 0.05 *N* sodium fluoride.

Nitrogen contents were determined by the micro-Kjeldahl method. The RNA, DNA and protein of the homogenates were separated by the method of Schmidt and Thannhauser (*6*).

RESULTS

Phosphorylase Activity of Tumors, Tumor Bearing Rat Liver and Normal Liver—The results are presented in Table II. There is no marked difference between the phosphorylase activity of normal rat and mouse liver, even after rats had been fasted for 24 hours. In rats with solid tumors (DAB-hepatoma and AH-130 hepatoma), the phosphorylase activity of the normal part of the liver decreases to nearly

TABLE II

Phosphorylase Activity of Tumor Cells, Tumor-Bearing Rat Liver and Normal Rat and Mouse Liver

Phosphorylase activity was assayed as described in the text.

	Strain of Rat or Mouse	Tumor	No. of Experiments	Phosphorylase Activity (μmoles iP/10 min./mg. Total Nitrogen)
Normal Liver	Sprague-Dawley rat	——	16	10.50±1.24[a]
	Sprague-Dawley rat[b]	——	3	10.10±2.14
	ddo-mouse	——	4	10.01±2.03
	C_3H-mouse	——	2	9.12±0.58
Tumor-Bearing Rat Liver	Sprague-Dawley rat	DAB-hepatoma (solid)	6	5.83±2.52
	Sprague-Dawley rat	AH-130-hepatoma (solid)	7	5.57±1.06
	Sprague-Dawley rat	AH-130-hepatoma (ascites)	5	10.00±3.06
Tumor Cells	Sprague-Dawley rat[c]	DAB-hepatoma	6	1.51±0.75
	Sprague-Dawley rat	AH-130-hepatoma (solid)	7	1.71±0.68
	Sprague-Dawley rat	AH-130-hepatoma (ascites)	9	2.92±1.01
	ddo-mouse	Ehrlich carcinoma	7	0.90±0.30
	C_3H-mouse	MH-129-D hepatoma	2	1.04±0.06
	(Tissue culture)	HeLa cells	2	2.19±0.33

a) standard deviation
b) fasted for 24 hours
c) host of tumor cells

half that in the normal liver, while in those rats bearing ascites tumor (AH-130 hepatoma) the phosphorylase activity of the liver is nearly normal.

The phosphorylase activity of tumor cells is very low, and as shown in Table II, activity differs from tumor to tumor. Ascites tumors of mouse (Ehrlich carcinoma and MH-129 D hepatoma) possess about one tenth the activity of normal mouse liver, as already shown by Nierenberg (*2*). However, in ascites tumor of rat (AH-130 hepatoma), the activity is one fifth to two fifths of that of normal liver. Solid tumors of rat (DAB- and AH-130-hepatoma) have one tenth to one fifth and HeLa cells one fifth to one fourth of the activity of normal liver.

Phosphorylase Activity of Regenerating Rat Liver—The change in phosphorylase activity of regenerating liver of Sprague-Dawley rats after partial hepatectomy is shown in Fig. 2. The phosphorylase activity decreases to about one half 100 hours after the operation and then gradually increases to normal.

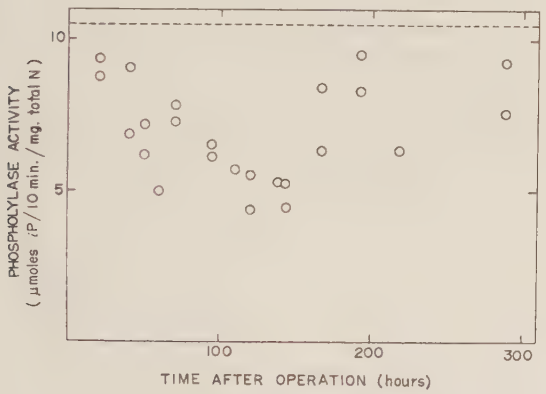

Fig. 2. Change in phosphorylase activity of regenerating liver after partial hepatectomy. The broken line represents the normal phosphorylase activity (see Table II). The activity was assayed as described in the text.

Phosphorylase Activity of Suckling Rat Liver—The phosphorylase activity of suckling rat liver is presented in Table III. The activity is very low especially during the first and second days after birth.

Table III

Phosphorylase Activity of Suckling Rat Liver

Phosphorylase Activity was assayed as described in the text.

Days after Birth	Phosphorylase Activity μmoles iP/10 min./mg. Total Nitrogen
0–1	1.20
0–1	0.50
0–1	0.30
1–2	1.73
2–3	6.32
2–3	6.23
5–6	5.43

DISCUSSION

The observation of Nierenberg (*2*) that the phosphorylase activity of tumors is very low in comparison with that of the liver of normal rats and mice, was confirmed. Furthermore it was found that rat liver with a high rate of cell division, such as regenerating liver and suckling rat liver, also possesses a low phosphorylase activity. While in Nierenberg's data the phosphorylase activity was expressed in μmoles of inorganic phosphate liberated per 10 minutes per mg. of protein

Table IV

Protein, RNA and DNA Contents of Tumor Cells and Normal Rat and Mouse Liver

Protein, RNA and DNA were separated by the method of Schmidt and Thannhauser (*4*). The nitrogen content was determined by the micro-Kjeldahl method.

	Nitrogen Content % of Total Nitrogen in Homogenate		
	Protein	RNA	DNA
Rat Liver	46.8	24.7	5.8
Mouse Liver	46.0	30.0	6.1
Ehrlich carcinoma	39.4	32.0	8.8
AH-130 ascites hepatoma	30.5	30.3	9.8
AH-130 solid hepatoma	32.1	34.3	9.3
DAB-hepatoma	32.9	27.3	7.5
MH-129 D hepatoma	38.9	34.6	9.8
HeLa cells	31.9	34.5	11.7

in the tissue homogenate, the values given here are expressed in μmoles of phosphate liberated per 10 minutes per mg. of total nitrogen in the homogenate. Table IV shows protein, RNA and DNA contents of homogenates of several tissues as a percentage of the total nitrogen. The protein content of tumor cells is lower than that of normal liver. Hence, if the activity is expressed per mg. of protein, the ratios of phosphorylase activity in tumors to that in normal liver in our experiments would be slightly larger than the present values.

Glycogen phosphorylase is one of the enzymes controlling carbohydrate metabolism. However, this enzyme may not be so essential for active cell division and growth in a restricted part of the body with a high metabolic rate, because this enzyme is not in the main path of glucose catabolism. Tumor tissue is a tissue with very high energy consumption and consequently glycogen phosphorylase would not be so essential to it. This explanation seems consistent with results on regenerating liver. In the present experiments 60 to 70 per cent of the liver was removed and 5 to 7 days after the operation regeneration was complete. Fig. 2 shows that the total phosphorylase activity is approximately constant during this period and that little or no synthesis of phosphorylase occurs during the first 140 hours. After the liver has been regenerated the synthesis of this enzyme begins.

SUMMARY

1. The glycogen phosphorylase activity of various tumors, including ascites and solid tumors, was measured and the finding of Nierenberg that this enzyme is considerably less active in tumors than in normal tissues was confirmed.

2. Tissues having a high rate of cell division, such as regenerating rat liver and suckling rat liver also have less phosphorylase activity than normal rat liver.

3. These results are discussed in relation to cell physiology.

The authors wish to express their gratitude to Prof. M. Kuru and Prof. S. Akabori for their guidance.

REFERENCES

(*1*) Nierenberg, M. W., *Biochim. et Biophys. Acta*, **30**, 203 (1958)

(*2*) Nierenberg, M. W., *J. Biol. Chem.*, **234**, 3088 (1959)

(*3*) Higgins, G. M., and Anderson, R. M., *Arch. Pathol.*, **12**, 189 (1931)

(*4*) Sutherland, E. W., and Wosilait, W. D., *J. Biol. Chem.*, **218**, 459 (1956)

(*5*) Takahashi, T., *Protein Nucleic Acid Enzyme* (Japanese), **2**, 464 (1957)

(*6*) Schmidt, G., and Thannhauser, J. S., *J. Biol. Chem.*, **161**, 83 (1945)

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Isolation, Characterization of An Abnormal γ-Glycoprotein from Urine of Patients with Cancer*

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Comparative studies on glycoproteins and neutral heteropolysaccharides of cancerous tissue, of body fluids of cancer bearing individuals and those from normals have long been a subject of our research group directed by late Dr. H. Masamune (*2–7*).

Of what it has been investigated by us, the following two findings were in particular noticeable; the qualitative change of blood group polysaccharides of cancerous tissue (*1–4*) and the appearance of the anemia inducing glycoprotein (*5–7*). Studies on biological properties of those muco-substances in attempting to correlate the biological abnormalities with their chemical or physical basis lead to an assumption that certain structure presumably is present in muco-substances of cancerous tissue in general, which was considered to be responsible for biological immaturity of cancerous tissue and the name 'immature structure' has been proposed, resting merely on the immunological similarities between the muco-substances of cancerous tissues and those of biologically immature tissue such as rapidly growing embryo or placenta. This appeared to be the first recognition of 'qualitative change' of muco-substance in connection with their biological significance, although little is known as to the chemical and physical basis of the biological abnormality.

On the other hand, pure chemical and physical comparison of serum α-glycoprotein of the cancer bearing rat with those of the normals were made by Nisselbaum and Bernfeld (*8*), although they were unable to find out definitive chemical basis of the abnormality, but pointed out that cancerous α-glycoprotein are much homogeneous on electrophoresis over a wide range of pH compared with that of normals and suggested that one of the normal component might be increased enormously. Turumi (*9*) obtained a glycoprotein from cancerous ascites which resembled to α_2-glycoprotein of serum, but claimed that the same glycoprotein was not obtained from tuberculous ascites. Nagai (*10*) attempted to correlate hexose/hexosamine ratio on the several buches of urinary glycoprotein similar to that of Anderson-Maclagen and noticed that hexosamines were abundant in cancerous fraction.

In contrast to those changes of higher molecular glycoproteins, we have recently noticed the presence of a low molecular dialyzable glycopolypeptide markedly increased in case of cancer but not in cases of inflamation, to which the name "κ-Mucopolypeptide" was given (*11–14*). It has been partly characterized (*12–13*) but still remained to be elucidated which is now under way.

Present report deals with a new systematic fractionation of urinary glycoprotein adsorable on benzoic acid (*15–16*), through which we have recognized a group of glycoproteins as the most carbohydrate rich but sialic acid poor fraction (Fr. 4-glycoproteins). A remarkable change in carbohydrate composition during canceration has been observed in glycoproteins of this fraction. After a number of trials, an abnormal glycoprotein has been

* 3rd report of "Urinary Mucoproteins and Mucopolypeptides in Cancer". Read before the 33rd General Meeting of the Japanese Biochemical Society, Oct. 31—Nov. 2, 1960 (*1*).

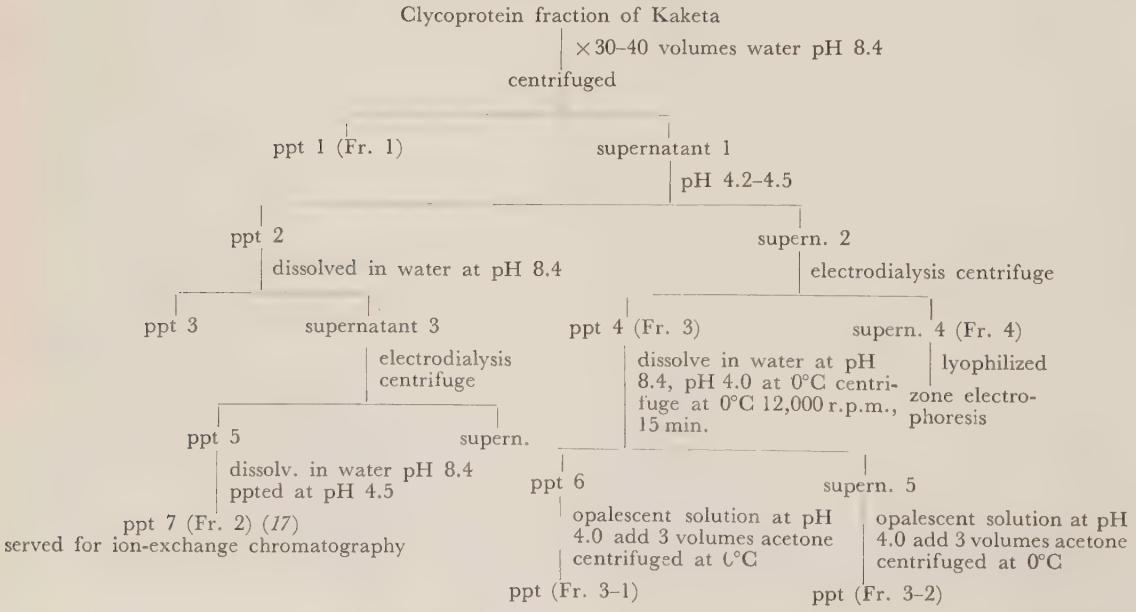
isolated therefrom by zone electrophoresis in an essentially homogeneous form both ultracentrifually and electrophoretically. This glycoprotein was shown to be one of the γ-glycoprotein as it moved with γ-globulin fraction in veronal or phosphate buffer but migrates more rapidly in sodium borate or Tris-borate system. The corresponding normal γ-glycoprotein has been also isolated from the corresponding normal Fr. 4-glycoproteins. The carbohydrate component irrespective of normal or pathological were identified as galactose, mannose, L-fucose, a pentose, glucosamine, galactosamine and sialic acid. The abnormal γ-glycoprotein is characterized as compared with the normal by the following outstanding feature; 1) greater molecular weight, 2) greater degree of branching and 3) less amount of hexoses compared with hexosamines. And those chemical and physical basis of molecular abnormalities have been further proved by observation of changes of the corresponding γ-glycoprotein fraction of the rat's urine during cancer development after being innoculated with Ascites Hepatoma 136, which will be published elsewhere (*16 a*). Thus it has increasingly been apparent that a certain definitive correlation exist between cancerration and systemic 'qualitative changes' of glycoprotein.

EXPERIMENTAL

Fractionation of the Benzoic Acid Adsobable Glycoprotein—1. The glycoprotein fraction co-precipitable with benzoic acid (I) was prepared according to Kaketa's (*7*) modification (The principle of the method was originally described by Remington on serum, the modified application of the fractionation on urinary glycoprotein was first described by Nakagawa and Kasai (*16*), later on by Anderson and Maclagan (*15*), The yield of the substance: approximately 5.0 g. from 10 liters of cancerous urine and 2.5 g. from the same volume of normal urine. The substance was fractionated according to Scheme 1, namely it was dissolved in 30–40 volumes (v/w) of water at pH 8.4, the pH was adjusted with 0.5 *N* NaOH under cooling in an ice bath and centrifuged (12,000r.p.m. 30 minutes). The precipitate was treated with the same volume of alkaline water at pH 8.4 for three times. The precipitate was washed with water and lyophilized (Fr. 1). The combined supernatant above (supern. 1) was acidified with 0.5 *N* HCl to pH 4.2–5, a bulky precipitate (ppt. 2) was spun down (3,000 r.p.m.) and the supernatant was separated (supern. 2). The ppt. 2

Scheme I

Fractionation of Urinary Glycoprotein Adsorable on Benzoic Acid



dissolved in water at pH 8.4 was electrodialyzed and the precipitate occurred on electrodialysis was again dissolved in water at pH 8.4 and precipitated at pH 4.2–5 and finally dried from frozen state (Fr. 2).

2. Fr. 2 was further purified on electrodialysis followed by ion exchange chromatography and analyzed as was described in the forgoing article (*17*).

3. The supernatant 2 was served for electrodialysis in the Pauly's apparatus for three days until the current reached less than 0.5 mA. A bulky precipitate occurred during electrodialysis was collected by centrifugation and freeze dried (ppt 4–Fr. 3). The yield was 773 mg. from 10 g. of cancerous "I" and 318 mg. form the same amount of normal "I". The precipitate can be further separated at 0°C into pH 4.0-precipitable (Fr. 3–1) and pH 4.0-soluble (Fr. 3–2) fraction by successive precipitation of the alkaline solution at pH 4.0, followed by acetone fractionation. Namely, Fr. 3 weighing 0.733 g. was dissolved in 36.6 ml. of water at pH 8.4 under cooling, insoluble part was rejected by centrifugation and the supernant fluid was acidified by 0.5 *N* H_2SO_4 to pH 4.0, centrifuged at 0°C 12,000 r.p.m., 15 minutes. The precipitate was dissolved in water at pH 8.4, followed by aciduation to pH 4.0. No precipitation but an opalescence occurred, which was precipitated by addition of three volumes of acetone and 1 ml. of a saturated sodium acetate in methanol and centrifuged. The precipitate was washed with methanol and dried (Fr. 3–1). The first supernatant was added with three volumes of acetone and 1 ml. of a saturated sodium acetate in methanol, centrifuged, dialyzed with water and lyophilized (Fr. 3–2). Yield; Fr. 3–1: 399.5 mg., Fr. 3–2: 302 mg. from 0.733 g. of Fr. 3 of cancerous source and Fr. 3–1: 147 mg., Fr. 3–2: 149.8 mg. from 0.3182 g. of normal source.

4. The soluble supernatant after the Fr. 3 being removed on electrodialysis was perevorated in a cellophane bag until about 100 ml. and lyophilized (Fr. 4). Yield: 1.5 g. from 15 g. of the normal "I" and 2.8 g. from 15 g. of the cancerous "I". Comparison of carbohydrate component of Frs. 2, 3 and 4 in respect to cancerous and normal are shown in Table I.

It is evident from these results that Fr. 4 shows strongest change in hexose/hexosamine ratio. Further fractionation on zone electrophoresis was achieved as it was shown to be inhomogeneous in Tiselius electrophoresis.

5. Fractionation of Fr. 4-glycoprotein on vertical zone electrophoresis at pH 8.4. Four hundred mg. of the Fr. 4 in one run was separated on ethanolized cellulose according to Flodin (*18*). Mesh of

Table I

Carbohydrate Composition of Various Glycoproteins Adsorable on Benzoic Acid

	Fr. 2-Glycoproteins		Fr. 3-Glycoproteins				Fr. 4-Glycoproteins	
			Fr. 3–1		Fr. 3–2			
Analysis	Can.	Norm.	Can.	Norm.	Can.	Norm.	Can.	Norm.
Hexoses[1]	5.1	4.3	4.8	3.7	6.8	6.2	8.5	12.5
Pentoses[1]	—	—	2.7	2.1	2.0	2.1	2.0	2.5
Methylpentoses[1]	0.5	0.5	2.3	1.8	1.6	1.8	2.1	2.5
Hexosamines[1]	2.7	2.9	5.1	5.3	7.4	6.9	8.5	8.6
Sialic acid[1]	3.6	4.4	5.2	5.1	7.8	7.9	3.2	3.8
Non Reducing[2] Terminals	—	—	1.3 (4.9)	1.3 (4.9)	1.0 (3.9)	1.9 (7.5)	2.8 (10.9)	2.1 (8.2)
Hexose/Hexosamine Ratio	1.9	1.5	0.9	0.7	0.9	0.7	1.0	1.4

1) Methods of analysis see the text; each numbers express the percentage of individual carbohydrate component to the dried, ash free specimen (dried *in vacuo* over $CaCl_2$). The percentages are expressed as follows; hexoses: as galactose, pentoses: as xylose, methylpentoses: as L-focose, hexosamines: as free glucosamine and sialic acid: as N-acetyl-neuraminic acid ($C_{11}H_{19}O_9N$).

2) Expressed as the percentage of the formic acid evolved on periodate oxidation to the dried specimen as above. In parenthesis are shown percentages of the non-reducing monosaccharide unit per total molecule, provided that one mole of formic acid is produced per one end group of carbohydrate chain, greater number of which indicate the greater degree of branching.

cellulose; 200–400, column size; 3.5×40 cm., buffer solution; pH 8.4 boric acid-sodium tetraborate (17.6 g. $Na_2B_4O_7$, 9.3 g. H_3BO_4 in 2 liters of water), temperature of run; below 10°C (7–10°C), condition of charge; 10 mA constant, 800–900 volts, duration; 36 hours.

Separation was reproducible as is shown in Fig. 1 when the conditions were well controlled as such. The fractions belonging to the same peak were combined, thoroughly dialyzed and lyophilized, thus glycoproteins of Fr. 4-P-1, Fr. 4-P-2, Fr. 4-P-3 and Fr. 4-P-4 were obtained in respect to the cancerous and to the normal. The carbohydrate component of those fractions are shown in Table II.

The most characteristic change for those fractions of cancerous sources seemed to be the lost amount of hexoses, which was most remarkable in Fr. 4-P-3-

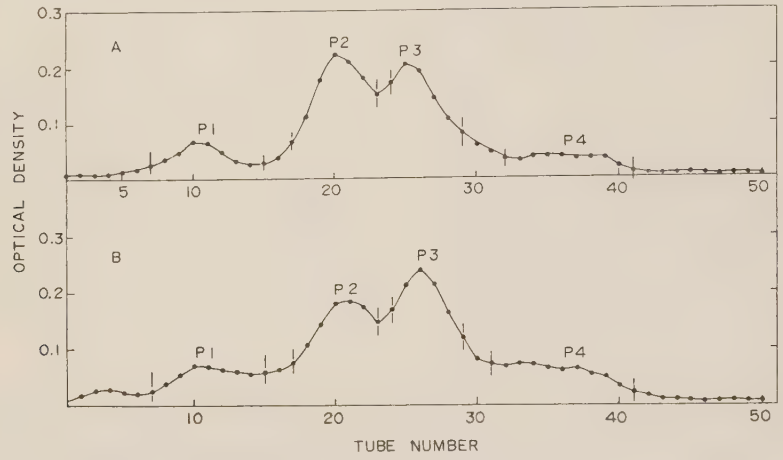


Fig. 1. Elution patterns of vertical zone electrophoresis of Fr. 4-Glycoproteins. A; Normal, B; Cancerous.

400 mg. of Fr. 4, dissolved in 0.05 *M* borate buffer pH 8.4 was put on the bufferized ethanolyzed cellulose column (40×4 cm.) within a width of 2–3 cm., 10 mA constant, 850–900 volts, for 36 hours at 7°–10°C.

After completion of electrophoresis, zones were eluated with the same buffer, aliquot of 3 ml. were collected by the fraction collector, from which 0.1 ml. was mixed with 0.4 ml. of water and 4.5 ml. of 89% sulfuric acid, heated in a boiling water bath for 3 minutes. Depth of coloration was read by Klett colorimeter 15 minutes after addition of 0.2 ml. of 1% α-naphthol.

Table II

Carbohydrate Composition of Fr. 4-Glycoproteins

	Fr. 4-P-1		Fr. 4-P-2		Fr. 4-P-3		Fr. 4-P-4	
	Can.	Norm.	Can.	Norm.	Can.	Norm.	Can.	Norm.
Hexoses[1)]	7.6	7.4	10.0	11.8	11.7	15.4	7.0	8.4
Pentoses[1)]	3.0	4.0	1.8	2.2	2.2	3.6	2.0	2.8
Methyl-pentoses[1)]	2.1	2.4	1.8	2.3	1.7	3.6	1.9	2.5
Hexosamines[1)]	7.3	4.6	7.7	8.3	9.5	10.3	4.6	5.0
Sialic acid[1)]	2.0	2.5	4.5	7.9	3.4	5.6	2.5	3.2
None reducing[2)] Terminals	—	—	2.4 (9.4)	1.8 (7.0)	2.1 (8.2)	1.5 (5.9·	1.3 (5.1)	0.5 (1.95)
Hexose/Hexosamine Ratio	1.0	1.6	1.3	1.4	1.2	1.5	1.5	1.7

1) Methods of analysis see the text; percent expressions of the individual carbohydrate are the same as is shown in Table I.

2. Percentage of the formic acid evolved on periodate oxidation and the none reducing monosaccharide unit calculated therefrom, those are the same as in Table I.

glycoprotein fraction and this tendency has become more remarkable on purification by reptition of zone-electrophoresis.

Thus the Fr. 4-P-3-glycoprotein was further purified on zone-electrophoresis at the same condition as described above. The substance analysed as is shown in Table III.

Methods of Analysis—Hexoses were determined by the method of Masamune-Sakamoto HR 10 (Thionalid-H_2SO_4) reaction (*19*), which gave constant and reliable results and all the aldohexoses gave nearly the same extinction. Hexosamines were determined according to Masamune and Yoshizawa's modification (*22*) of Elson-Morgan reaction and glucosamin/galactosamine ratio were determined according to Masamune and Yoshizawa (*22*). Pentoses were determined according to Masamune-Sakamoto (*20*) PR-2 reaction, which was not completely satisfactory but gives reliable results without serious variation if dichromatic reading at 392 and 435 mμ was taken. Methylpentose was determined according to Dische-Shettle's (*21*) method. Sialic acid was determined by orcinol method of Böhm *et al.* (*23*) (since the direct Ehrlich method has been experienced to be unreliable by unspecific coloration). In all these determinations, a simple mixture of sample solution in varying concentration of sulfuric acid, heated for varying duration of times is taken as the blank run and the reaction mixture of various monosaccharides from 10 to 100 μg. was run as the standard. Determination of formic acid on periodate oxidation was based on the Meyer's principle (*24*) with the following scale. As the origical method was not described in details, the method has been adapted for the present aim, namely 14–15 mg. of the substance dissolved in 5 ml. of water were mixed with 2 ml. of 0.1 *M* $NaIO_4$, let it stand at dark for 12 hours. Afterwards the mixture was added with ethylen glycol (8%, 1 ml.), allowed to stand for 30 minutes, followed by addition of 0.5 ml. of 0.5 *M* KI and 3 ml. of 0.01 *N* $Na_2S_2O_3$ in turn, and back titrated with 0.01 *N* I_2, starch as indicator. As the reference run, mixture of 0.5 ml. or 1.0 ml. of 0.01 *N* HCOOH in 5.0 ml. of water were added with 0.1 *M* $NaIO_4$ and 1 ml. of 8% ethylen glycol in turn and titrated with 0.01 *N* I_2 after addition of 0.5 ml. of 0.5 *M* KI and 3 ml. of 0.01 *N* $Na_2S_2O_3$. As the blank run, 15 mg. of the substance in 5.0 ml. of water were mixed with the mixture of 2 ml. of 0.1 *M* $NaIO_4$ and 1 ml. of 8% ethylene glycol which had been prepared in advance, and similarly treated and titrated.

The α-hydroxy-amino group was determined according to Aminoff *et al.* (*25*) employing Conway's diffusion analysis. One ml. of 2% boric acid containing two drops of brom cresol green and methyl red (a. a. 0.5%) were pipetted into inner chamber, the substances weighing 3.0 mg. were taken in outer chamber, dissolved in 0.5 ml. of water and to it were added 0.1 ml. of 9% (w/v) glycine, 0.25 ml. of 1 *N* NaOH, 0.2 ml. of 1 *M* HIO_4 and 1 ml. of saturated K_2CO_3 in turn without delay, and the units were quickly closed. They were incubated at 20°C for 24 hours. The same amount of the substance were incubated in the same way without addition of HIO_4 as the control run. The inner fluid of the unit was carefully titrated with 0.01 *N* NaOH by micropipett.

The volatile acid produced on hydrolysis were analyzed according to the Mizukami's modification (*26*) of Friedrich Repport method and expressed as the acetyl value.

Paper chromatography was used for the identification of component sugars; the following solvent systems in descending run were used: 1) ethylacetate, acetic acid, pyridin, water (5 : 1 : 5 : 3 v/v) as the developing solvent and ethylacetate, pyridine, water (40 : 11 : 6 v/v) at the bottom of the chamber in which hexosamines were separated very clearly during 24 hours. 2) Butanol, pyridine, water (10 : 3 : 3 v/v) in which glucose, galactose and mannose were separated very clearly during 48 hours. 3) Butyl-acetate, acetic acid, methanol, water (5 : 3 : 1 : 2 v/v). Hydrolysis with 1 *N* H_2SO_4 for 5 hours was employed. As the developer, silver nitrate method of Travelyan *et al.* (*27*) and aniline hydrogen phthalate method (*28*) were employed.

Optical rotation and the rotational dispersion was measured in Hitachi spectrophoto-electric polarimeter as the 1% solution.

Electrophoretic patterns were taken in Hitachi apparatus (Schlieren-diagonal method) as the 1% solution in 0.2 *M* veronal buffer of pH 8.6 ($\Gamma/2=0.1$), phosphate buffer of pH 7.7 ($\Gamma/2=0.1$), borate buffer of pH 8.4 (the same buffer as was used in zone-electrophoresis) and Tris-EDTA-boric acid buffer of pH 9.0 (*28a*).

Sedimentation patterns of the most purified specimens were taken in Spinko Model E. The patterns of Fr. 4-P-3 glycoproteins irrespective of cancerous and normal were proved to be essentially homogeneous although it contained similar but slightly different degree polymerization, as the single peak descended in increased breadth of peak. As to the condition of the experiments see the foot-note of Fig. 3.

Molecular weight of the most purified Fr. 4-P-3-glycoproteins were measured according to the osmotic pressure methode of Bull and Currie (*29*) 1% and 0.5% solution at 29.5°, duration of the time for equilibration: 3–4 hours.

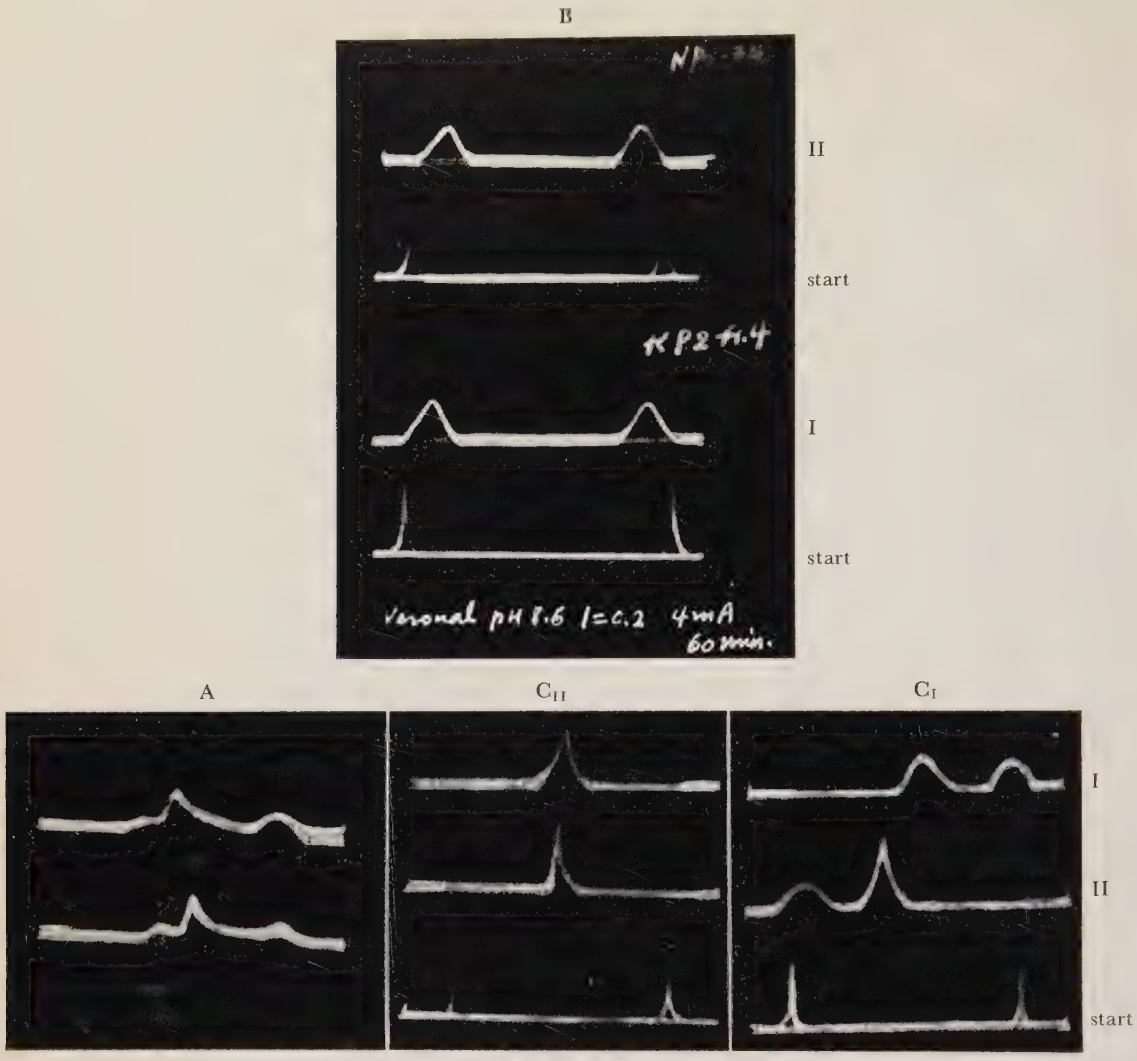


Fig. 2. Electrophoretic patterns of various glycoprotein fraction.

A: 0.8% solution of Fr. 4-Glycoproteins in Tris-EDTA-boric acid buffer of pH 9.0, $\Gamma/2=0.65$. I: Cancerous, II: Normal. 4 mA, 45 minutes after start, 20°C.

B: 1% solution of Fr. 4-P-3-Glycoproteins (κ-Glycoproteins) of cancerous (I) and normal (II) in 0.2 *M* veronal buffer of pH 8.6, $\Gamma/2=0.1$ 4 mA, 60 minutes after start, 20°C.

C: 0.8% solution of Fr. 3-glycoproteins of cancerous (I) and normal (II) in Tris-EDTA-boric acid buffer of pH 9.0, $\Gamma/2=0.65$, 4 mA.

RESULTS

Characterization of Fr. 2-Glycoprotein—This is essentially the same as the glycoprotein described as the 'toxohormone active without KIK,' which was already reported by us (*17*). The amount of the carbohydrate was lowest of all the fraction and the ratio hexose/hexosamine was lower in normals than in the cancerous, which was the same tendency as was reported by Nagai (*10*).

Characterization of Fr. 3-Glycoprotein—Both Fr. 3-1 and Fr. 3-2 are characterized by that the amount of hexosamines exceeds that of hexoses as is shown in Table I. On electrophoresis, as is shown in Fig. 2, no essential

difference is found as to the mobilities of the main peaks between cancerous and normal, but the cancerous fraction sometimes contains a large second component as the stationary peak (Fig. 2 C_1).

The bioassay of Fr. 3–1 and Fr. 3–2 revealed that the former fraction contains strong 'amemia inducing' and '*in vivo* catalase depressing' activities, namely this fraction seems to be the identical glycoprotein that Kaketa (7) had described. Further characterization will appear elsewhere.

Characterization of Fr. 4-Glycoproteins—This fraction is characterized by that the amount of hexoses exceeds that of hexosamines as is shown in Table II. The most characteristic change of this fraction for cancerous sources seems to be the lost hexose residue, which is most remarkable in Fr. 4-P-3-glycoprotein and this tendency has become more pronounced on purification by repitition of zone-electrophoresis. The final specimen was proved to be homogeneous on electrophoresis and on ultracentrifuge and was considered to be a γ-glycoprotein as was mentioned in the introductory remark. This γ-glycoprotein of cancerous sources are completely different from the glycoprotein of normal sources not only in chemical composition but also in molecular weight and optical rotational dispersion. It is considered, therefore, that this glycoprotein of cancerous sources must be regarded as an abnormal γ-glycoprotein. The greatest difference in hexosamine content was observed, however, in the fastest migrating Fr. 4-P-1

Table III

Chemical Composition and Physical Properties of Abnormal and Normal γ-Glycoproteins

	Abnormal γ-Glycoprotein (Fr. 4-P-3)	Normal γ-Glycoprotein (Fr. 4-P-3)
Nitrogen[1]	10.4	7.1
Protein Moiety[2]	62.5	52.0
Hexoses[3]	11.0	16.0
Pentoses[3]	2.5	7.5
Methylpentoses[3]	2.4	3.3
Hexosamines[3]	9.0	8.5
Glucosamine	5.5	5.0
Galactosamine	3.3	3.2
Sialic acid[3]	4.1	3.5
None-reducing[4] Terminals	2.5 (9.8)	1.2 (4.7)
Volatile Acid[5]	5.0	4.8
α-Hydroxy Amino-[6] Group	0.33	0.75
Hexose/Hexosamine Ratio	1.22	1.95
None Reducing[7] terminals per Total Carbohydrate	25	10
Electrophoretic mobilities 10^{-5}cm²/sec. volt	−1.05	−1.10
Molecular Weight	3.2×10^4	0.8×10^4

1) Micro-Kjeldahl. 2) Calculated from the total nitrogen minus amino-sugar nitrogen. 3) 4) Expressions and calculations are the same as described at the foot-note of Table I. 5) Percent of the volatile acids as the acetyl. 6) Per cent of the ammonia evolved on periodate oxidation. 7) Per cent of the terminal monosaccharide unit to the total carbohydrate moiety, greater number of which indicates the greater degree of branching.

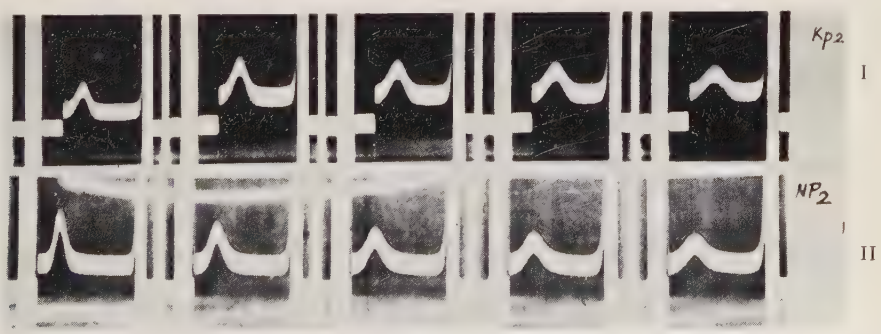


Fig. 3. Ultracentrifuging patterns of 0.5% solution in water of Fr. 4-P-3-Glycoproteins of cancerous (I) and normal (II), at 20°C. 15 minutes intervals from right to left. 45,000 r.p.m.

and that in the degree of branching was in the stationary component. Those fractions though interesting were not studied in detail as the yields were low, which will be studied in future.

On the basis of analytical results of carbohydrate component and molecular weight and other physical properties of more than ten different specimens of Fr. 4-P-3-glycoprotein preparation of different cancer and those of the normals, it has been confirmed that the following most outstanding changes were considered to be the common phenomena of the glycoprotein during canceration.

1. Formation of the larger molecule, three to four times as much as the normal molecule.

2. Hexoses are always decreased, hexosamines remains unchanged thus hexose/hexosamine ratio decreased in cancer.

3. Degree of branching, measured as formic acid evolution on periodate oxidation, are twice as much as the normal molecule.

The glucosamine/galactosamine ratio and the amount of sialic acid are unchanged. Those chemical and physical basis of the abnormality of the molecule have been further confirmed by observation of changes of rat's glycoprotein during the course of cancer development which will be appeared elsewhere (*16 a*).

The rotatory dispersion of Fr. 4-P-3 and the changes on denaturation in 8 *M* urea and on enzymatic hydrolysis as to Fr. 4-P-3 glycoprotein are shown in Fig. 4. A striking positive shifting of the curve of cancerous glycoprotein at the wave length shorter than 480 mμ must be a "Cotton effect". It is, however, noteworthy that the difference between concerous and normal are changed to show parallel curve after complete denaturation with 8 *M* urea and the difference is lost after being digested with streptomyces proteinase, which is considered that the difference in rotatory dispersion might be attributed to the difference of helical structure of polypeptide moiety due to the sequential difference of amino acids.

DISCUSSION

The experimental results indicate an obvious alteration of normal Fr. 4-P-3 glycoprotein into the abnormal glycoprotein during canceration in not only chemical composition but also molecular size and feiner structural figures.

Although an interesting conformational difference indicated by rotational dispersion has been recognized, the eventual conclusion should await to be drawn because of lackness of fundamental knowledge of helical structure and optical rotations in groups of carbohydrate moiety of the molecule and because of a prescence of unusual "positive shifting" for

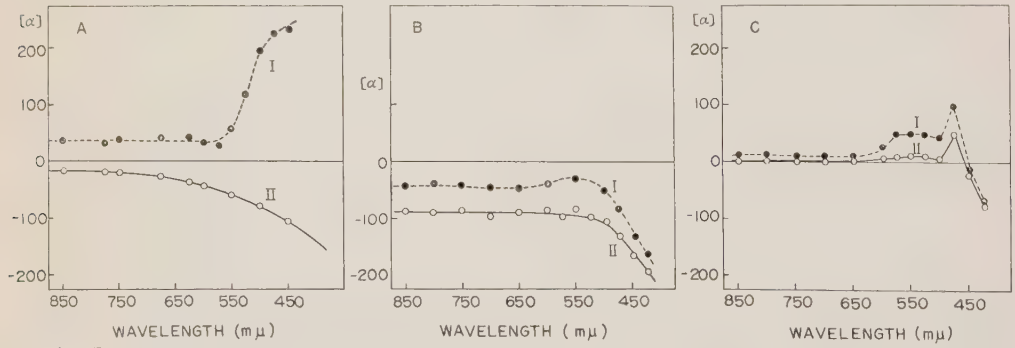


Fig. 4. Rotational dispersion curves of Fr. 4-P-3-Glycoproteins of Cancerous (I) and normal (II).
A: Before treatment, B: After complete denaturation with 8 *M* urea, C: After enzymatic hydrolysis with "Pronase" (KAKEN).
1% solution in water in A, in 8 *M* urea in B and in water containing 0.02% Pronase in C. Read in Hitachi spectrophotometric polarimeter in 10 cm. tube at room temperature (15°C in A, 18°C in B and C).

a protein, which might be due to a certain chromophilic grouping. An ultimate conclusion will be appeared in sometime elsewhere, nevertheless changes of molecular weight, degree of branching and of hexose/hexosamine ratio have now been recognized, as essential molecular changes consistently occur during cancer development. The idealized version of those molecular changes is illustrated in Fig. 5.

Fig. 5. Idealized version on the molecular change of γ-Glycoprotein.

Left: three small molecules of normal γ-glycoprotein.

Right: one large molecule of abnormal γ-glycoprotein.

Black balls represent aminosugars including sialic acid, white balls represent none amino-sugars and curved strands represent polypeptide chains. The version emphasizing increased degree of branching, increased molecular weight and increased proportion of amino-sugars in the carbohydrate moiety of abnormal molecule.

The change of hexose/hexosamine ratio was first noticed by Nagai (*10*) who claimed that hexosamines decreased and the ratio increased during canceration of a glycoprotein fraction similar to that of Anderson-Maclagan. This tendency is true on Fr. 2-glycoprotein from present investigation although the value is rather irregular from one preparation to another. Contrary to those, the ratio is always decreased during canceration in all preparation of Fr. 4-glycoproteins, whereby the amount of hexosamines remains unchanged and the none amino sugar decreases. Namely, a certain structure made up of none-amino sugar, which is considered to be a responsible structure of normal physiological function, must have been lost during canceration,

In view of the present recognition of an important significance of hexose/hexosamine ratio and the degree of oligosaccharide branching of the molecule of blood group polysaccharide and other muco-substances in determination of their specificity (*30–31*), significance of present findings of the molecular abnormality of cancerous glycoprotein must be a reflection of a lost specificity of the normal molecule and of a renovate appearance of a certain structure responsible for the pathological specificity, which is characterized by a higher degree of branching and by a decreased amount of hexoses.

Further studies on immunological and biological significance of this abnormal glycoprotein in special connection to cancer-host relationship is now being under way.

SUMMARY

1. A systematic fractionation of high molecular urinary glycoproteins adsorable on benzoic acid has been developed and the four distinctive groups of glycoproteins were distinguished according to the carbohydrate content, amount of hexosamine, hexose and sialic acid. Each group is consisted of similar but different glycoproteins.

2. The most soluble, carbohydrate rich but sialic acid poor fraction (Fr. 4) exhibits most significant changes in cancer bearing individuals, from which an abnormal glycoprotein characteristic to cancer has been isolated in almost homogeneous form (Fr. 4-P-3-glycoprotein or κ-glycoprotein), which is considered to be one of the γ-glycoprotein.

3. κ-Glycoprotein has been characterized on comparison with the normal γ-glycoprotein by the following chemical and physical properties: a) 3–4 times greater molecular weight, b) about 2–2.5 times greater degree of branching, c) lost hexoses, namely decreased hexose/hexosamine ratio.

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REFERENCES

(*1*) Hakomori, S., Kawauti, H., and Ishimoda, T., *J. Japan. Biochem. Soc.*, **32**, 656 (1960)
(*2*) Oh-uti, K., *Tohoku J. Exp. Med.*, **51**, 297 (1949)
(*3*) Masamune, H., Kawasaki, H., Abe, S., Oyama, K., and Yamaguchi, Y., *Tohoku J. Exp. Med.*, **68**, 81 (1958)
(*4*) Kawasaki, H., *Tohoku J. Exp. Med.*, **68**, 119 (1958); **69**, 153 (1959)
(*5*) Masamune, H., and Kawasaki, H., *Tohoku J. Exp. Med.*, **63**, 369 (1956)
(*6*) Sugimoto, K., *Tohoku J. Exp. Med.*, **64**, 271 (1956)
(*7*) Kaketa, H., *Tohoku J. Exp. Med.*, **67**, 319 (1958)
(*8*) Nisselbaum, J. S., and Bernfeld, P., *J. Am. Chem. Soc.*, **78**, 687 (1956)
(*9*) Turumi, K., *Fukushima J. Med. Sci.*, **3**, 31 (1956)
(*10*) Nagai, Y., *Fukushima J. Med. Sci.*, **3**, 13 (1956)
(*11*) Masamune, H., Hakomori, S., and Sugo, T., *Tohoku J. Exp. Med.*, **69**, 383 (1959)
(*12*) Masamune, H., and Hakomori, S., *Gann*, **50**, *Suppl.* 108 (1959)
(*13*) Masamune, H., and Hakomori, S., *Symposium on cytochemistry*, **10**, 206, Maruzen Publ. Co. Tokyo. (1959)
(*14*) Hakomori, S., *Protein, Nucleic acid Enzyme*, **4**, No. 6, 14 (1959)
(*15*) Anderson, A. J., and Maclagan, N. F., *Biochem. J.*, **59**, 638 (1955)
(*16*) Nakagawa, S., and Kasai, A., *Nippon Naikagakkai Zasshi* (*J. Japan. Intern. Med.*) **40**, 312 (1951)
(*16a*) Hakomori, S., Kawauti, H., and Ishimoda, T., *Nature*, in press.
(*17*) Masamune, H., Hakomori, S., Kaketa, H., and Sinohara, H., *Tohoku J. Exp. Med.*, **68**, 63 (1958)
(*18*) Flodin, P., and Kupke, B. W., *Biochim. et Biophys. Acta*, **21**, 368 (1956)
(*19*) Masamune, H., and Sakamoto, M., *Tohoku J. Exp. Med.*, **63**, 345 (1956)
(*20*) Ibidem, ibid., **63**, 357 (1956)
(*21*) Dische, Z., and Shettles, L. B., *J. Biol. Chem.*, **175**, 595 (1948)
(*22*) Masamune, H., and Yoshizawa, Z., *Tohoku J. Exp. Med.*, **65**, 286 (1954)
(*23*) Böhm, P., Dauber, St., and Baumeister, L., *Klin. Wschr.*, **32**, 286 (1954)
(*24*) Meyer, K. H., *Advances in Enzymology*, **3**, 109 (1943)
(*25*) Aminoff, D., and Morgan, W. T. J., *Biochem. J.*, **48**, 74 (1951)
(*26*) Mizukami, S., Ieki, T., and Koyama, Ch., *J. Pharm. Soc. Japan*, **76**, 465 (1956)
(*27*) Travelyan, W. E., Procter, D. P., and Harrison, J. S., *Nature*, **166**, 444 (1950)
(*28*) Partridge, S. M., *Nature*, **164**, 443 (1949)
(*28a*) Aronsson, T., and Grönwall, A., *Scand. J. Clin. Lab. Invest.*, **9**, 339 (1957)
(*29*) Bull, H. B., and Currie, B. T., *J. Am. Chem. Soc.*, **68**, 742 (1946)
(*30*) Hiyama, N., Fukushima, Y., and Takahashi, T., *J. Japan. Biochem. Soc.*, **30**, 1069; **31**, 109 (1959)
(*31*) Gibbons, R. A., Morgan, W. T. J., and Gibbons, M., *Biochem. J.*, **60**, 428 (1955)

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Studies on the Respiratory Enzyme Systems of Plants

II. The Oxidation of Reduced Diphosphopyridine Nucleotide by the Subcellular Preparations of Rice Plant Seedlings*

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(Received for publication, December 8, 1960)

It is generally believed that the terminal respiration in plant tissues is catalyzed, as in the case of animal tissues, by the particulate electron-transport systems which are characterized by the presence of cytochrome components (*1*, *2*). Plant cells, on the other hand, are unique in that they possess powerful peroxidatic activities (*3*, *4*). Very little is, however, known of the physiological functions of peroxidases in the plant metabolism.

In the present investigation it was aimed to obtain information concerning the pathways by which DPNH** is oxidized by molecular oxygen in the subcellular preparations obtained from rice-plant seedlings, a material which has never been studied with respect to the electron-transport systems. It was thus shown that entirely different mechanisms are functioning in the mitochondrial and soluble fractions for the oxidation of DPNH. The mitochondrial oxidation seems to be catalyzed by the ordinary cytochrome mechanism, whereas that in the soluble fraction appears to be mediated by a system involving a peroxidase.

EXPERIMENTALS

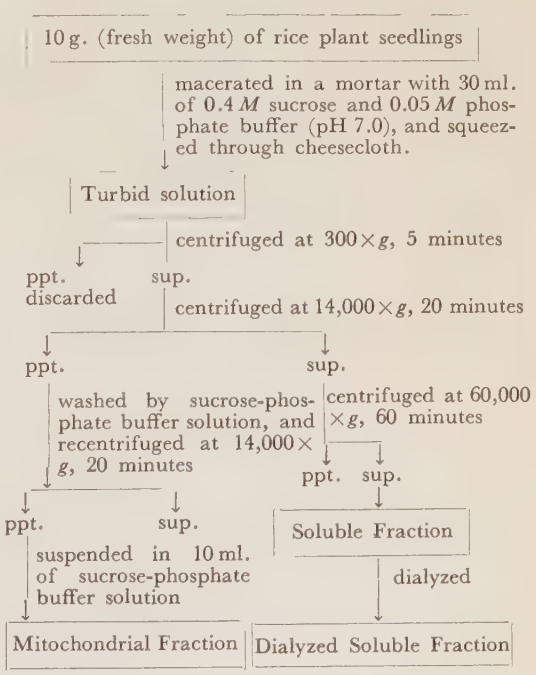
Rice-Plant Seedlings—The seeds of rice plant, variety Noorin No. 29, were suspended in a NaCl solution having a density of 1.10, and only those seeds which sedimented in this medium were used for the experiments. The seeds thus selected were sterilized with a 0.1% solution of Uspulun for 5 hours, and then allowed to germinated in a large Petri dish in the dark at 28°C. After 3 to 4 days, the seedlings, both tops and roots, were separated from the endosperms by a pincette, and subjected to the fractionation procedures.

Preparations of Subcellular Fractions—The fractionation of the seedlings was carried out according to the flow-sheet summarized in Scheme I. All the operations were conducted at temperatures not exceeding 4°C. Dialysis against water was carried out overnight in a refrigerator using a cellophane tubing.

Boiled Soluble Fraction—The soluble fraction (un-

Scheme I

Flow-sheet of Centrifugal Fractionation of Rice Plant Seedlings



* Part of this work was presented at the 33rd Annual Meeting of the Japanese Biochemical Society, Tokyo, November, 1, 1960.

** Reduced diphosphopyridine nucleotide is abbreviated as DPNH.

dialyzed) was boiled for 10 minutes, cooled to room temperature, and centrifuged at 10,000×*g* for 20 minutes. The supernatant thus obtained was used as the boiled soluble fraction.

Cytochrome c—The preparation by Nutritional Biochemical Company was used.

Crystalline Peroxidase—Crystalline Japanese radish peroxidase a (JRP-a) (*5*) was kindly supplied by Dr. Y. Morita of Kyoto University.

Antimycin A—The crystalline preparation kindly supplied from the Kyowa Fermentation Industries, Ltd. was dissolved in ethanol, and diluted to desired concentrations with distilled water before use.

DPNH Oxidation Assay—The oxidation of DPNH was determined by following the decrease in optical density at 340 mμ using a Hitachi EPU spectrophotometer. The cuvette was kept at about 20°C. The reaction mixture usually consisted of 0.5 ml. of enzyme, 1.0 ml. of 0.1 *M* phosphate buffer (pH 7.0), 0.5 ml. of $4.4\times10^{-4}M$ DPNH, and distilled water or other additions to a final volume of 4.0 ml. The reaction rate was expressed in terms of the decrease in optical density per 5 minutes (during the interval of 1 to 6 minutes after the addition of DPNH) per mg. nitrogen of enzyme preparation. When the rate was too high, the values observed during shorter intervals (2 or 3 minutes) were converted to 5 minutes by calculation.

Oxygen Uptake Assay—Oxygen uptake was measured at 30°C using conventional Warburg manometric techniques. DPNH was tipped in after 10 minutes equilibration. The reaction mixture consisted of 1.0 ml. of $7.2\times10^{-3}M$ DPNH, 1.0 ml. of 0.1 *M* phosphate buffer (pH 7.0), and 1.0 ml. of mitochondrial fraction (total N, 0.63 mg.) or 2.0 ml. of soluble fraction (total N, 0.89 mg.), respectively. In the case of the soluble fraction, phosphate buffer was not added. The measurement was carried out at 30°C.

Determination of Total Nitrogen—The amount of total nitrogen of the subcellular preparations was determined by micro-Kjeldahl method after digestion by sulfuric acid.

RESULTS

Effects of Added Cytochrome c—As can be seen from Fig. 1, the oxidation of DPNH by the rice-plant mitochondrial fraction was found to be considerably stimulated by the adddition of cytochrome c prepared from mammalian heart muscle. The oxidation by the soluble fraction was, however, rather inhibited by added cytochrome c as is shown in Table I.

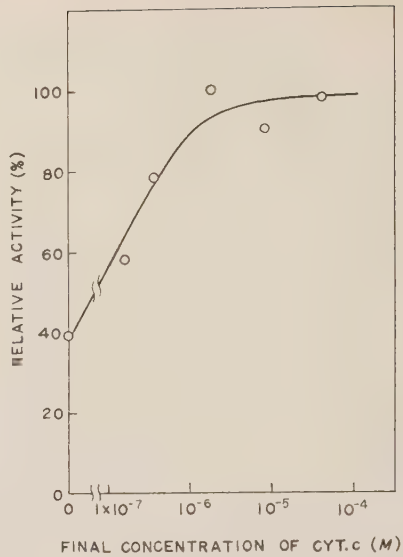


FIG. 1. Effect of cytochrome c on DPNH oxidation by mitochondrial fraction.

Reaction mixture: 0.5 ml. of $5.5\times10^{-4}M$ DPNH, 1.0 ml. of 0.1 *M* phosphate buffer (pH 7.0), 0.5 ml. of mitochondrial fraction (N, 165 μg.), and cytochrome c; total volume 4.0 ml.

TABLE I

Effect of Cytochrome c on DPNH Oxidation by Soluble Fraction

Addition	Δ O.D. at 340 mμ./5 min. /mg. N of enzyme	Inhibition per cent
Control	0.478	—
Cytochrome c[1]	0.259	46

1) Final concentration of cytochrome c was $2.5\times10^{-5}M$.

TABLE II

Effect of Cytochrome c and Resorcinol on DPNH Oxidation by Dialyzed Soluble Fraction

Additions	Δ O.D. at 340 mμ./5 min. /mg. N of enzyme	Inhibition per cent
Control	0.15	—
Resorcinol[1]	4.58	—
Resorcinol and cytochrome c	1.16	75

1) Final concentrations of resorcinol and cytochrome c were $1\times10^{-4}M$ and $1.25\times10^{-5}M$, respectively.

As will be described in a later section of this paper, the soluble fraction, when dialyzed, oxidized DPNH only when supplemented with the boiled soluble fraction, resorcinol, phenol, *etc.* Cytochrome c was also inhibitory to the resorcinol-activated oxidation of DPNH by the dialyzed soluble fraction (Table II).

Effects of Respiratory Inhibitors—Table III shows that the oxidation of DPNH by the mitochondrial fraction in the absence of added cytochrome c is sensitive to both cyanide and antimycin A.

Table III

Inhibitory Effects of Cyanide and Antimycin A on DPNH Oxidation by Mitochondrial Fraction

Inhibitors	Δ O.D. at 340 mμ./5 min./mg. N of enzyme		
	A without cytochrome c	B with cytochrome c[1]	C B−A
None	0.525	0.976	0.451
$1\times10^{-3}M$ CN⁻	0.142(73)[2]	0.255(74)	0.113(75)
5 μg./ml. Antimycin A	0.210(60)	0.568(42)	0.358(21)

1) Final concentration of cytochrome c was $5\times10^{-6}M$.

2) Numerals in brackets show per cent inhibition.

Most of the increase in the activity caused by the addition of cytochrome c was, however, insensitive to antimycin A, though it was still inhibited by cyanide. The oxidase activity of the soluble fraction was, in contrast to that of the mitochondrial fraction, not at all inhibited by antimycin A (Table IV), but was quite sensitive to cyanide (Table VI).

Table IV

Effect of Antimycin A on DPNH Oxidation by Soluble Fraction

Additions	Relative activity in per cent
None	100.0
0.5% Ethanol	99.4
5 μg./ml. Antimycin A	101.3

The mitochondrial activity, both in the presence and absence of added cytochrome c, was found to be inhibited not only by cyanide but also by azide, though the inhibition by azide was less pronouced than that by cyanide (Table V). Unlike these results obtained with the mitochondrial fraction, the oxidation of DPNH by the soluble fraction was completely insensitive to azide in spite of the strong inhibition caused by cyanide (Table VI). These facts strongly suggested that a peroxidase is

Table V

Inhibitory Effects of Azide and Cyanide on DPNH Oxidation by Mitochondrial Fraction

Inhibitors	Δ O.D. at 340 mμ./5 min./mg. of N of enzyme	
	without cytochrome c	with cytochrome c[1]
None	0.310	0.649
$1\times10^{-3}M$ Azide	0.161(48)[2]	0.299(54)
$1\times10^{-3}M$ CN⁻	0.103(67)	0.230(65)

1) Final concentration of cytochrome c was $5\times10^{-6}M$.

2) Numerals in brackets show per cent inhibition.

Table VI

Effects of Azide and Cyanide on DPNH Oxidation by Soluble Fraction and by Crystalline Peroxidase

Additions	Δ O.D. at 340 mμ./5 min./mg. of N of enzyme		
	Soluble fraction	Soluble fraction +Resorcinol	JRP-a[1] +Mn^{++} +Resorcinol
None	0.823	1.580	207
$1\times10^{-3}M$ Azide	0.865(−5)[2]	1.983(−25)	198(4.4)
$1\times10^{-3}M$ CN⁻	0.080(91)	0.084(94)	4(98.1)

1) JRP-a: Japanese radish peroxidase a. The amount of nitrogen of peroxidase was calculated from its optical density at 405 mμ. (*5*).

2) Numerals in brackets show per cent inhibition; those with minus sign per cent activation.

involved in the oxidation of DPNH by the soluble fraction, since it had been shown by Akazawa and Conn (*6*) that horse

radish peroxidase can actively oxidize DPNH by molecular oxygen if supplemented with Mn^{++} and certain phenolic compounds and that this oxidation is sensitive to cyanide but not to azide. As included in Table VI, the oxidation of DPNH by crystalline peroxidase from Japanese radish in the presence of Mn^{++} and resorcinol was found to be inhibited by cyanide but by azide in agreement with the results obtained by Akazawa and Conn (*6*).

Effects of Redo- and Oxidogenic Substances—Peroxidases of plant origin have been shown to oxidize various compounds such as DPNH (*6*), dihydroxyfumaric acid (*7*), and indoleacetic acid (*8*) in the absence of hydrogen peroxide. Such oxidase activities of peroxidases are either activated or inhibited by certain phenolic compounds, *etc.* Yamazaki (*9*) classified these compounds into two groups, the redogenic and oxidogenic substrates. The former inhibits and the latter activates the oxidase activity. Table VII shows some of the redo- and oxidogenic substrates as classified by Yamazaki (*9*).

TABLE VII

Classification of the Substances Which Affect the Oxidation of Dihydroxyfumaric Acid and Triose Reductone etc. by Peroxidase (*9*)

Redogenic substrates	Oxidogenic substrates
Indoleacetic acid	*p*-Cresol
Ascorbic acid	*m*-Cresol
Hydroquinone	Guaiacol
Pyrogallol	Resorcinol
p-Phenylenediamine	*m*-Phenylenediamine
Catechol	Aniline
	Uric acid

Since the results described above suggested the participation of a peroxidase in DPNH oxidation by the soluble fraction, it was decided to study the effects of these redo- and oxidogenic substances on the DPNH oxidase activities of both mitochondrial and soluble fractions.

(*a*) *Mitochondrial Fraction*—As is shown in Table VIII, the oxidation of DPNH by the mitochondrial fraction was considerably increased by the addition of resorcinol. This

TABLE VIII

Effects of Resorcinol, Catechol, and Cytochrome c on DPNH Oxidation by Mitochondrial Fraction

Additions	Relative activity in per cent
DPNH alone	100
Resorcinol[1)]	193
Catechol	102
Cytochrome c	155
Resorcinol and Catechol	106
Resorcinol and Cytochrome c	179

1) Final concentration of each additions was $1\times10^{-4}M$, except for cytochrome c whose concentration was $5\times10^{-6}M$.

activation by resorcinol was completely abolished by the addition of catechol, although catechol itself had no effect on the oxidation. The activity in the presence of resorcinol and cytochrome c was lower than that with resorcinol alone. It seems that either the activation by cytochrome c is inhibited by resorcinol or the activation by resorcinol inhibited by cytochrome c.

(*b*) *Soluble Fraction*—Table IX shows that

TABLE IX

Effects of Various Chemicals on DPNH Oxidation by Soluble Fraction

Additions	Relative activity in per cent
DPNH alone	100
Resorcinol[1)]	286
Phenol	378
α-Naphthylamine	303
Ascorbic acid	140
p-Phenylenediamine	200
Hydroquinone	186
Catechol	25
Cytochrome c	54
Resorcinol and catechol	48

1) Final concentration of each additions was $1\times10^{-4}M$, except for cytochrome c ($5\times10^{-6}M$).

the oxidation of DPNH by the soluble fraction is markedly accelerated by the addition of resorcinol, phenol, α-naphthylamine, ascorbic acid, hydroquinone, and *p*-phenylenediamine. Among these substances, however, ascorbic acid, hydroquinone and *p*-phenylenediamine inhibited the activation by resorcinol. Catechol, pyrogallol, phloroglucinol and cytochrome c, on the other hand, were rather strong inhibitors of the DPNH oxidation. Catechol also abolished the activation due to resorcinol. Manganous ion was found to activate the oxidation.

(*c*) *Dialyzed Soluble Fraction*—The results obtained with the soluble fraction could be more clearly confirmed with the dialyzed soluble fraction. As can be seen from Table X, the dialyzed fraction could hardly oxidize DPNH by itself. The oxidase activity could, however, be markedly activated by the addition of resorcinol. Manganous ion, though by itself failed to activate the oxidation in this case, greatly stimulated the DPNH oxidation in the presence of resorcinol. Phenol also showed similar effects as resorcinol. Of considerable interest is the fact that the boiled soluble fraction acted as the activator. The study of the active principle in the boiled fraction responsible for this activation seems of importance to evaluate the physiological functions of this oxidation. The stimulative actions of resorcinol, phenol and the boiled fraction were inhibited by catechol; catechol itself had no activating effect on the activity of dialyzed soluble fraction.

Hydroquinone, *p*-phenylenediamine and ascorbic acid also had activating effects, although their effects were less than those due to resorcinol and phenol. Furthermore, the rate of DPNH oxidation in the presence of

Table X

Effects of Various Chemicals on DPNH Oxidation by Dialyzed Soluble Fraction

Additions	Relative activity in per cent
DPNH alone	3.3
Resorcinol (R.)[1]	100.0
Phenol	75.8
Catechol (C.)	3.9
Mn^{++}	7.5
Hydroquinone (H.Q.)	39.0
p-Phenylenediamine (*p*-PDA)	42.3
Ascorbic acid (V. C.)	40.5
R. and C.	6.6
R. and H. Q.	29.3
R. and *p*-PDA	34.4
R. and V. C.	23.6
R. and Cytochrome c	20.9
R. and Mn^{++}	465.
Boiled fraction (1.0 ml.)	62.3
Boiled fraction (0.5 ml.)	34.0

1) Final concentration of each additions was $1\times10^{-4}M$, except for cytochrome c ($1.25\times10^{-5}M$). Indicated volume of boiled fraction was added to a total volume of 4.0 ml.

Table XI

Effects of Various Chemicals on DPNH Oxidation by Crystalline Japanese Radish Peroxidase a

Additions	Relative activity in per cent
DPNH alone	0.7
Resorcinol (R.)[1]	1.9
Mn^{++}	0.7
Resorcinol and Mn^{++}	100.0
Phenol and Mn^{++}	28.3
Catechol and Mn^{++}	6.9
Hydroquinone and Mn^{++}	4.3
R., Mn^{++} and Catechol	14.4
R., Mn^{++} and Hydroquinone	5.1
R., Mn^{++} and Cyt.	68.5
Boiled fraction	193.0 (100)
Boiled fraction and H. Q.	74.6[2] (39)
Boiled fraction and Catechol	4.4 (2)

1) Final concentration of each additions was the same as in Table X, except for the boiled fraction. 0.5 ml. of the boiled solution was added to a total volume of 4.0 ml.

2) The rate of the DPNH oxidation in the presence of both boiled fraction and hydroquinone becomes nil 5 minutes after the start of reaction.

resorcinol was considerably lowered by the simultaneous addition of these compounds. It is likely that these effects of hydroquinone, *etc.* are in some way related to the laccase activity of the soluble fraction and will be considered in more details in Discussion.

(*d*) *Crystalline Peroxidase*—As mentioned earlier, Akazawa and Conn (*6*) found that horse radish peroxidase is able to oxidize DPNH in the presence of both Mn^{++} and resorcinol. This was confirmed in the present study using a crystalline preparation of Japanese radish peroxidase a. As will be seen from Table XI, phenol was found to replace resorcinol to some extents. Catechol and hydroquinone had little activation effects even in the presence of Mn^{++}. They in fact inhibited the resorcinol-activated oxidation as did cytochrome c. The boiled soluble fraction behaved similarly to resorcinol, but in this case the presence of Mn^{++} had no effects on the oxidation.

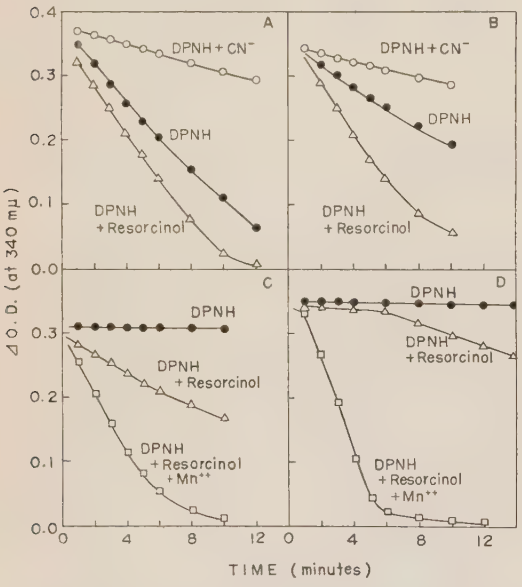


FIG. 2. The time course of DPNH oxidation. A: mitochondrial fraction (N, 153 μg.); B: soluble fraction (N, 120 μg.); C: dialyzed soluble fraction (N-content was the same as B. before the dialysis.); D: crystalline Japanese radish peroxidase (N: ●, 7.0 μg.; △, 3.5 μg.; □, 1.75 μg.). Final concentration of CN^- was $1\times10^{-3}M$, resorcinol and Mn^{++} $1\times10^{-4}M$, respectively.

Time Course of DPNH Oxidation—In Fig. 2 are illustrated the time course of DPNH oxidation observed under selected conditions by four different enzyme systems, *i.e.* the mitochondrial fraction, the soluble fraction, the dialyzed soluble fraction, and crystalline Japanese radish peroxidase a.

Oxygen Uptake in DPNH Oxidation—Since all the experiments described above were conducted by spectrophotometrically measuring the disappearance of DPNH, it was felt necessary to manometrically confirm that the observed oxidation was in fact accompanied by the consumption of molecular oxygen. Typical results of such manometric experiments are shown in Fig. 3. The data plotted in this figure were obtained by substracting the endogenous oxygen uptake from the observed values. The endogenous consumption of oxy-

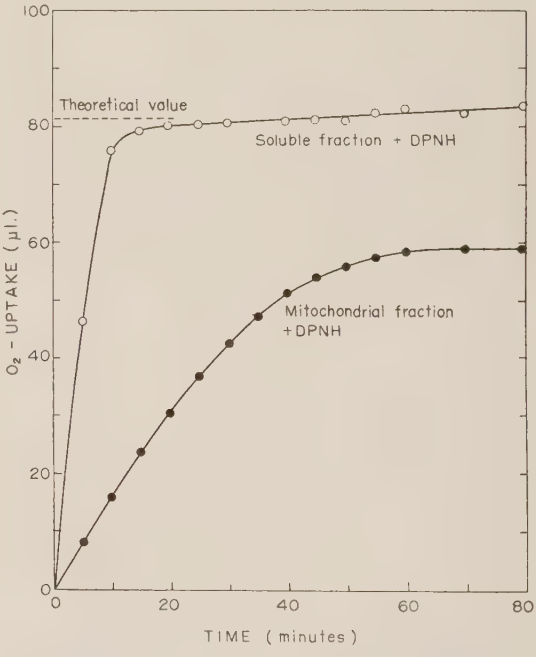


FIG. 3. Oxygen-consumption in DPNH oxidation by mitochondrial and soluble fraction.

Reaction mixture: 1.0 ml. of $7.2\times10^{-3}M$ DPNH; 1.0 ml. of 0.1 *M* phosphate buffer (pH 7.0); and 1.0 ml. of mitochondrial fraction (N, 0.63 mg.) or 2.0 ml. of soluble fraction (N, 0.89 mg.), respectively; total volume, 3.0 ml. In the case of soluble fraction, phosphate buffer was not added. Temperature, 30°C.

gen in the mitochondrial system was considerably high and sometimes reached about one half of the total consumption, whereas that in the soluble fraction was usually negligible.

DISCUSSION

The effects of various substances tested in the present study on the oxidation of DPNH

TABLE XII

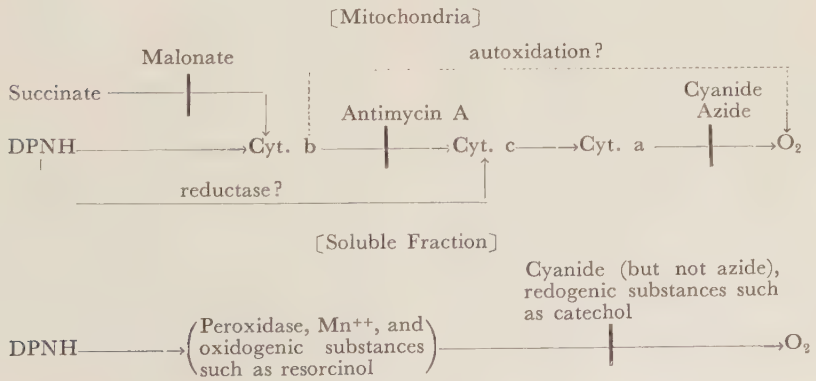
Summary of the Effects of Various Additions on DPNH Oxidation

Additions	Mitochondrial fraction	Soluble fraction	Dialyzed soluble fraction	Crystalline peroxidase
DPNH alone	+	+	0	0
Mn^{++}			0	0
Resorcinol (R.)	+	+	+	0
Hydroquinone (H. Q.)		+	+	
Catechol	0	−	0	
Cytochrome c	+	−	0	
R. and Mn^{++}		+	+	+
R. and H. Q.		−	−	−(with Mn^{++})
R. and Catechol	−	−	−	−(with Mn^{++})
R. and Cytochrome c	− or 0		−	−(with Mn^{++})
Boiled fraction			+	+
Boiled fraction and catechol			−	−
Inhibition by CN^-	+	+	+	+
Inhibition by azide	+	−	−	−
Inhibition by antimycin A	+	−	−	−

R. and H. Q. are representatives of the groups consisting of resorcinol, phenol, α-naphthylamine, and β-naphtylamine; and of hydroquinone, p-phenylenediamine, and ascorbic acid, respectively. In upper three colums, + shows activating, and − inhibitory actions by the additions, and 0 means that the addition has no significant effect on DPNH oxidation. In the last colum, the inhibitory action is shown by +, and − means that the addition has no inhibitory activity.

SCHEME II

Tentative Schemes for the Electron Transport Pathways of the Subcellular Preparations of Rice Plant Seedlings



catalysed by the subcellular preparations from rice-plant seedlings as well as by Japanese radish peroxidase a may be summarized as shown in Table XII. It appears that most of these data can be sufficiently explained by assuming the electron-transport pathyways illustrated in Scheme II. That the oxidation of succinate by the mitochondrial fraction is inhibited by malonate was confirmed in an experiment which is not described in this paper.

In this scheme it is assumed that the main portion of DPNH oxidation in the mitochondrial fraction is catalysed by the ordinary cytochrome system which is inhibited by antimycin A (between cytochrome b and c) and by both cyanide and azide (between cytochrome a and oxygen). The mitochondria can also oxidize DPNH by an antimycin A-insensitive pathway if cytochrome c is added to the system. This oxidation is, however, sensitive to cyanide and is probably due to the antimycin A-insensitive DPNH-cytochrome c reductase followed by the oxidation of reduced cytochrome by the main pathway. A small fraction of the mitochondrial oxidation of DPNH is sensitive neither to antimycin A nor to cyanide. The slow autoxidation of cytochrome b may be responsible for this type of oxidation. There is no doubt that the ordinary cytochrome system is most important from the standpoint of energy metabolism, since it is most likely that only the electron transport through this system is coupled with the formation of adenosine triphosphate.

The fairly active oxidation of DPNH by the soluble fraction is explained in the scheme as caused by the action of a peroxidase which requires both Mn^{++} and oxidogenic substances such as resorcinol for the oxidation. The functioning of this system can be readily concluded from the similarity between the DPNH oxidation catalyzed by the soluble fraction, both dialyzed and undialyzed, of rice-plant seedlings and that catalysed by crystalline peroxidase prepared from horse radish (*6*) and Japanese radish. The fact that the soluble fraction and the dialyzed soluble fraction possess ordinary peroxidatic activities as measured with guaiacol and hydrogen peroxide lends further support to this conclusion. This type of DPNH oxidation has also been suggested by several workers (*4*, *10*, *11*).

As to the Mn^{++} requirement of peroxidases for the oxidase activity, we have previously reported that the oxidation of α-keto acids in plant juice is promoted by the addition of Mn^{++} (*12*). Kentén and Mann (*13*) have also found that the oxidation of oxalic and and oxaloacetic acids by horse radish peroxidase is stimulated by Mn^{++}. The concentration of Mn^{++} required for these reactions is, however, considerably higher than that needed for the DPNH oxidation by the soluble fraction. The oxidation of α-keto acids seems to resemble the oxidation of indoleacetic acid by horse radish peroxidase in this respect (*9*). Such discrepancies in the Mn^{++} levels required have also been pointed out by Akazawa and Conn (*6*). No satisfactory explanations can be given to this fact, but it should be emphasized here that the physiological level of Mn^{++} in the soluble fraction is sufficient for the oxidation of DPNH by the peroxidase system.

The effects of phenolic and other substances on the DPNH oxidation by the soluble fraction of rice-plant seedlings are beautifully in accord with Yamazaki's classification (*9*) of these substances into the redo- and oxidogenic substrates except for hydroquinone, *p*-phenylenediamine and ascorbic acid. These latter substances, though belonging to the redogenic or inhibitory substances, activate rather than inhibit the oxidation when they are added alone. This anormaly seems to be explicable if it is realized that these substances are readily oxidized by laccase whose presence in the soluble fraction was confirmed by a preliminary experiment. It does not seem unreasonable to assume that hydroquinone and *p*-phenylenediamine added to the soluble fraction are oxidized to *p*-benzoquinone or its analogue by the action of laccase, and the *p*-benzoquinone produced in turn oxidizes DPNH either enzymatically or nonenzymatically to regenerate hydroquinone. The oxidation of DPNH by *p*-benzoquinone

has been shown to occur in a plant system (*14*). Ascorbic acid, on the other hand, can act as the substrates for both laccase and ascorbic acid oxidase, and the latter enzyme is also contained in the soluble fraction of rice-plant seedlings. The ascorbic acid-activated oxidation of DPNH in the rice plant as well as the similar reaction in the potato tuber (*15*) is, therefore, an interesting problem left for future researches.

It is difficult at present to explain the fact that cytochrome c behaves like redogenic substrates in the oxidation of DPNH by the soluble fraction. It appears that further studies with various preparations of cytochrome c are needed before something can be said on this phenomenon.

The stimulating effect of the boiled fraction on the oxidation of DPNH catalyzed by both the dialyzed soluble fraction and crystalline Japanese radish peroxidase is of considerable interest from the physiological point of view. Akazawa and Conn (*6*) have reported that the phenol requirement of horse radish peroxidase for the DPNH oxidase activity can be substituted by boiled extracts from certain plants, but not by those from other plants. The failure of boiled extracts from the latter group of plants to substitute the phenol requirement may be due to the absence of activators in these plants, but it seems more likely that this is caused as a result of the balance between the content of redogenic (inhibitory) substrates and that of oxidogenic (activating) substrates. If this is the case, any metabolic changes in plant cells influencing the levels of redo- and oxidogenic substrates may be regarded as regulatory mechanisms for the oxidation of DPNH in the soluble cell fraction. It is interesting in this connection to recall the findings reported by Mudd *et al.* (*16*) that the content of inhibitors of the indoleacetic acid oxidation in quack-grass rhizomes undergoes seasonal variation. It will be an interesting problem of plant physiology to study the balance of redo- and oxidogenic substrates in various tissues of a plant species as well as in different species of plants and to follow its changes due to the physiological conditions, growth stages, *etc.* We have previously reported that the application of gibberellin, a plant growth regulator, causes an increase in the level of peroxidase activity in the rice plant (*17*). It seems, however, desirable to reevaluate this finding from the standpoint of the balance of inhibitors and activators.

The fact that the mitochondrial oxidation of DPNH can also be activated by resorcinol requires some comments. This activation does not seem to be related to the intrinsic properties of the mitochondrial system. Instead, it is more likely that the activation is due to the contamination or adsorption of peroxidase onto the mitochondria, since the washings of mitochondria were found to oxidize DPNH according to the peroxidase type of mechanism. The less pronounced inhibition of the mitochondrial oxidation by azide as compared with that by cyanide may be explained in the same way.

Attention may also be paid to the fact that α-naphthylamine activates the oxidation of DPNH by the soluble fraction, since the α-napthylamine-oxidizing activity is widely used in this country as a means of diagnosing the general activity of rice plant roots (*18*, *19*). It has also be shown that α-naphthylamine can peroxidatically oxidized by hydrogen peroxide in the presence of an extract from rice-plant roots (*20*). It is, therefore, probable that there exist some interrelationships between the oxidation of α-naphthylamine in rice-plant roots and its activation effect on the peroxidase-catalyzed oxidation of DPNH.

Finally, some discussions on the action of antimycin A may be pertinent from the standpoint of agronomy. This antibiotic is quite effective against the pathogenic fungus of rice blast, *Pilicuralia oryzae* (*21*). It has, however, no toxity toward the rice plant (*21*), and therefore is an ideal fungicide against the rice blast fungus, although its practical application in paddy fields is restricted its due to toxity toward fishes. Such selective toxity of antimcyin Ato the fungus has been ascribed to the fact that the electron-transport system of *P. oryzae* is composed of ordinary cytochromes a, b and

c, a system sensitive to antimycin A (*23*). The results reported in this paper, however, indicates that the oxidation of DPNH by the mitochondrial fraction of rice-plant seedlings is also very sensitive to the antibiotic. This is not in agreement with the resistance of rice plant to antimycin A. In preliminary experiments it was shown that the respiration of intact rice plant seedlings is scarecely inhibited by the antibiotic. Such discrepancies may be accounted for by assuming either the impermeability of intact rice-plant tissues to the antibiotic or the occurrence of antimycin A-insensitive respiration in the intact plant. These possibilities may not be overlooked in a search for effective fungicides and bacteriocides applicable for the rice plant.

SUMMARY

The oxidation of DPNH by the mitochondrial and soluble fractions prepared from rice-plant seedlings was surveyed, and the following results were obtained.

1. The mitochondrial fraction can oxidize DPNH, and this oxidation is inhibited by antimycin A, azide and cyanide. Mammalian cytochrome c activates the oxidation, but the increase due to cytochrome c is insensitive to antimycin A. It appears that the main portion of the mitochondrial oxidation is catalyzed by the ordinary cytochrome systems involving cytochromes a, b and c.

2. The soluble fraction can also oxidize DPNH. This oxidation is inhibited by cyanide and cytochrome c, but quite insensitive to antimycin A and azide.

3. The soluble fraction, when thoroughly dialyzed, loses its ability to oxidize DPNH. The lost activity can, however, be restored by the addition of boiled soluble fraction. The addition of resorcinol and other oxidogenic substrates together with Mn^{++} is also effective in activating the oxidation activity of dialyzed preparation.

4. From comparisons with the DPNH oxidase activity of crystalline Japanese radish peroxidase, it is concluded that the oxidation of DPNH in the soluble fraction is caused by the action of a peroxidase. There is a possibility that some of the oxidation is also catalyzed by a system involving laccase.

5. The oxidation of DPNH in both the mitochondrial and soluble fractions accompanies the consumption of molecular oxygen.

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REFERENCES

(*1*) Hackett, D. P., *Ann. Rev. Plant Physiol.*, **10**, 113 (1959)
(*2*) Hartree, E. F., *Advances in Enzymol.*, **18**, 1 (1957)
(*3*) Theorell, H., "The Enzyme" Vol. **II**, ed. by Sumner, J. B., and Myrbäck, K., Academic Press, New York, p. 397 (1951)
(*4*) Tagawa, K., and Shin, M., *J. Biochem.*, **46**, 865 (1959)
(*5*) Morita, Y., and Kameda, K., *Mem. Res. Inst. Food Sci., Kyoto Univ.*, No. **12**, 14 (1957)
(*6*) Akazawa, T., and Conn, E. E., *J. Biol. Chem.*, **232**, 403 (1958)
(*7*) Theorell, H., and Swedin, B., *Naturwissenshaften*, **27**, 95 (1939)
(*8*) Kenten, R. H., *Biochem. J.*, **59**, 110 (1955)
(*9*) Yamazaki, I., "Proceedings of the International Symposium on Enzyme Chemistry" Tokyo and Kyoto, Maruzen Co., Ltd., Tokyo, p. 227 (1958)
(*10*) Romberger, J. A., *Plant Physiol.*, **34**, 589 (1959)
(*11*) Sisler, E. C., and Evans, H. J., *Tabacco Sci.*, **147**, (1958)
(*12*) Murakami, Y., and Matsunaka, S., *Symposium on Enzyme Chemistry*, **12**, 94 (1957)
(*13*) Kenten, R. H., and Mann, P. J. G., *Bichem. J.*, **53**, 498 (1953)
(*14*) Wosilait, W. D., and Nason, A., *J. Biol. Chem.*, **205**, 255 (1954); Wosilait, W. D., Nason, A., and Terrell, A. J., *J. Biol. Chem.*, **206**, 271 (1954)
(*15*) Hackett, D. P., *Plant Physiol.*, **33**, 8 (1958)
(*16*) Mudd, J. B., Johnson, B. G., Burris, R. H., and Buchholtz, K. P., *Plant Physiol.*, **34**, 144 (1959)
(*17*) Hayashi, T., Murakami, Y., and Matsunaka, S., *Bull. Agr. Chem. Soc. Japan*, **20**, 159 (1956)
(*18*) Sakai, H., and Yoshida, T., *Res. Bull. Hokkaido Nat. Agr. Exptl. Sta.*, No. **72**, 82 (1957)
(*19*) Yamada, N., and Oota, Y., *Noogyogijitsu*, **13**, 435 (1958)

(20) Matsunaka, S., *J. Biochem.*, **47**, 820 (1960)
(21) Nakayama, K., Okamoto, F., and Harada, Y., *J. Antibiotics, Ser. A.*, **9**, 63 (1956)
(22) Misato, T., Ishii, I., Asakawa, M., and Fukunaga, K., *Ann. Phytopathological Soc. Japan*, **23**, 219 (1958)
(23) Matsunaka, S., *Symposium on Enzyme Chem.*, **15**, in press.

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Studies on the Denaturation of Enzymes

III. Effects of Anions on Heat Inactivation of Sweet Potato β-Amylase

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The rate of the heat denaturation of proteins or of the heat inactivation of enzymes varies with pH, kind and concentration of buffer, concentration of enzyme protein or the presence of various inorganic or organic substances.

In the course of the studies on the stability of sweet potato β-amylase, it was observed that various inorganic and organic ionic substances increased the rate of the heat inactivation of the enzyme. And it was considered that it might be a non-specific action of the charge of ions that instabilized the amylase (*1*).

In this paper, the effects of several species of anion upon the rate of the heat inactivation of sweet potato β-amylase were studied in some detail, and the relationship between the rate of inactivation and the charge of ion was discussed.

MATERIALS AND METHODS

Sweet Potato β-Amylase—Crystalline amylase obtained from dried powder of sweet potato (*1*), was recrystallized five times, and dialyzed against deionized water. This preparation was electrophoretically homogeneous.

In some experiments, however, the enzyme preparation was the same as used in the previous experiments (*1*, *2*).

The concentration of enzyme was expressed as mg. of protein nitrogen per ml. of solution, which was estimated by micro-Kjeldahl method. One mg. of protein nitrogen corresponds to 6.62 mg. of protein (*3*).

The Method of Heating Enzyme and the Measurement of Amylase Activity—The method of heating enzyme solution and the measurement of residual amylase activity were essentially the same as described in the previous paper (*2*), with the exception that the temperature of activity measurement was 35°C, and that the measurement of increase in reducing power of reaction mixture, was carried out by Somogyi's titration or colorimetric method (*4*). And the first order rate constant of inactivation was calculated from initial and residual activities.

The temperature of heat inactivation was 63± 0.1°C, and pH was 5.4 throughout the experiments.

RESULTS

Influences of Inorganic Salts upon Heat Inactivation of Sweet Potato β-Amylase—It was reported previously that the rate of heat

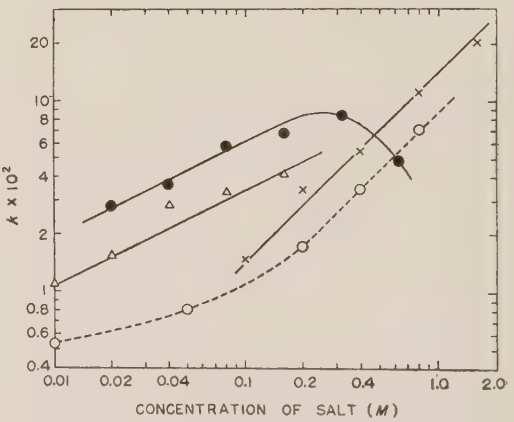

Fig. 1. Relationship between the rate constant of the amylase inactivation and the concentration of inorganic salt.

Enzyme solution containing *M*/50 acetate buffer (pH 5.4) and each salt was heated at 63± 0.1°C. Enzyme concentration, *cf.* Table I.

—×—: NaCl; —●—: Na_2SO_4; —△—: Na_2HPO_4; – – ○ – –: NaCl, data from the previous paper (*1*).

inactivation of sweet potato β-amylase increased in the presence of inorganic neutral salts, and that the more concentrated the salts, the more rapid was the inactivation. These experiments were repeated in wider range of salt concentration, and the relationships between the rate constants of enzyme inactivation and the salt concentrations were illustrated in Fig. 1.

In a certain range of the concentration of salt, logarithm of rate constant was linear to logarithm of salt concentration. This relationship can be expressed by the following equation,

$$k = k' [\mathrm{I}]^{\frac{1}{n}} \qquad (1)$$

where [I] is the molar concentration of salt. The slops, i/n, appeared to be a reciprocal of valence of anion, *e.g.* 1 for chloride and 1/2 for sulfate and dibasic phosphate.

At the higher concentration of sulfate, however, the rate of inactivation decreased.

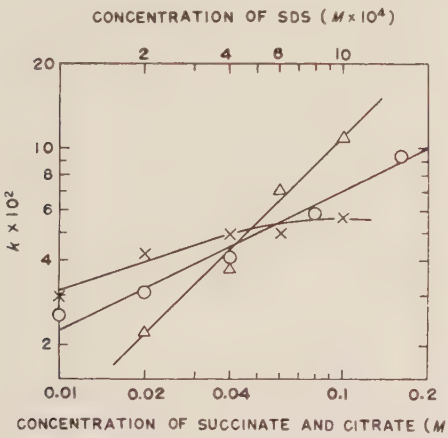

Fig. 2. Relationship between the rate constant the and concentration of organic salt.

Experimental conditions, same as in Fig. 1, except SDS, for which condition was the same as in the previous paper (*1*).

—○—: Succinate; —×—: Citrate; —△—: SDS.

Influences of Organic Acids upon the Rate of Heat Inactivation—Similar experiments were carried out with organic salts, sodium succinate and sodium citrate, and also with sodium laurylsulfate (SDS). Results, illustrated in Fig. 2, showed that, in the moderate concentration of salts, the relationships between the inactivation rate constants and the salt concentrations were similar to that with inorganic salts described above, where the slopes were approximately 1/2, 1/3 and 1 for succinate, citrate and SDS respectively. And the decrease of inactivation rate at higher concentration of salt was observed also in citrate under these experimental conditions.

DISCUSSION

In the previous paper (*1*) it was reported that the rate of heat inactivation of sweet potato β-amylase was increased in the presence of various ionic substances, of which effects appeared to be non-specific to the kind of substances.

Moreover, when organic acid (formate, acetate, succinate, malate or citrate) was added to the enzyme solution, its effect to increase the inactivation rate was approximately proportional to the basicity of the acid independent of its structure.

From these observations it was assumed that the function of these substances to accelerate the heat inactivation of sweet potato β-amylase may be due to their ionic charge.

In the present study, in which the effects of several inorganic and organic ionic substances upon the rate of heat inactivation of sweet potato β-amylase were investigated in more detail, it was found that Equation 1 was established between first order rate constant of inactivation and the concentration of the substances under these experimental conditions (Fig. 1 and 2).

Now, if an ion combines with one molecule of enzyme protein with its one unit of charge, so that the enzyme protein becomes unstable, and is inactivated more rapidly than is free enzyme, the inactivation reaction will be expressed as follows:

$$\mathrm{E} \xrightarrow{k_1} \mathrm{E}' \qquad (2)$$

$$\mathrm{E} + \tfrac{1}{n}\,\mathrm{I} \overset{\mathrm{K}}{\rightleftharpoons} \mathrm{EI}_{\frac{1}{n}} \xrightarrow{k_2} \mathrm{E}' + \tfrac{1}{n}\,\mathrm{I} \qquad (3)$$

where E, E′ and I are the active and inactive enzyme and ion of n valence respectively.

Equation 2 represents the inactivation of free enzyme, and Equation 3 that of enzyme-ion complex, in which k_1 and k_2 are first order rate constant of each reaction, and k_2 is considerably larger than k_1.

If total enzyme concentration is [E], and equilibrium constant of combining reaction is K, the concentration of enzyme-ion complex will be

$$[EI_{\frac{1}{n}}]=K([E]-[EI_{\frac{1}{n}}])\,[I]^{\frac{1}{n}}, \qquad (4)$$

for ion is present in much excess.
Therefore,

$$[EI_{\frac{1}{n}}]=\frac{K[E]\,[I]^{\frac{1}{n}}}{1+K[I]^{\frac{1}{n}}} \qquad (5)$$

Velocity of inactivation then will be expressed as

$$v=k_1([E]-[EI_{\frac{1}{n}}])+k_2[EI_{\frac{1}{n}}]$$

$$=\frac{k_1+k_2K[I]^{\frac{1}{n}}}{1+K[I]^{\frac{1}{n}}}\cdot[E]=k[E], \qquad (6)$$

where k is the rate constant of overall reaction. Therefore,

$$k=\frac{k_1+k_2K[I]^{\frac{1}{n}}}{1+K[I]^{\frac{1}{n}}} \qquad (7)$$

Provided that k_1 is so small that it is negligible as compared with $k_2K[I]^{\frac{1}{n}}$, reciprocal form of Equation 7 will become

$$\frac{1}{k}=\frac{1}{k_2K}\cdot\frac{1}{[I]^{\frac{1}{n}}}+\frac{1}{k_2} \qquad (8)$$

If $1/k$ is plotted against $1/[I]^{\frac{1}{n}}$, a straight line will be obtained, and the intercept and slope will be $1/k_2$ and $1/k_2K$ respectively.* Fig. 3 illustrates these relationships. And values of k_2 and K obtained by such treatment were summarized in Table I.

Since the value of k_1 is of an order of 10^{-3} or less, the rate constant k_2 is extraordinarily large, and the equilibrium constant K is considerably small except SDS. Therefore, in the range of an average concentration of ions, k_1 and $K[I]^{\frac{1}{n}}$ will be regarded as negligible as compared with $k_2K[I]^{\frac{1}{n}}$ and 1 respectively, then Equation 7 will be approximately equal to Equation 1 which was obtained experimentally.

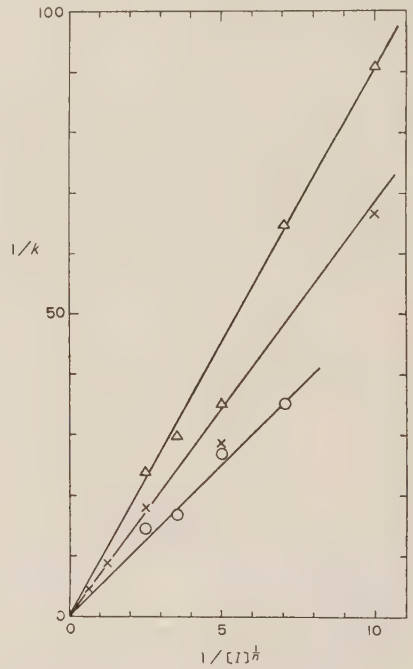

FIG. 3. Relationship between $1/k$ and $1/[I]^{\frac{1}{n}}$. —×—: NaCl; —○—: Na_2SO_4; —△—: Na_2HPO_4.

But at lower concentration of salt, $k_2K[I]^{\frac{1}{n}}$ will become so small that k_1 can not be neglected. The upward curve for chloride at lower concentration illustrated by dotted line in Fig. 1 may be the case. In fact k in the presence of 0.01 M chloride was only about 10 per cent larger than that in its absence.

It is noted that all salts tested have approximately the same value of k_2. This may be consistent with our previous sugges-

* Equation 7 can be rearranged as

$$k-k_1=\frac{(k_2-k_1)K[I]^{\frac{1}{n}}}{1+K[I]^{\frac{1}{n}}},$$

the reciprocal form of this is,

$$\frac{1}{k-k_1}=\frac{1}{(k_2-k_1)K}\cdot\frac{1}{[I]^{\frac{1}{n}}}+\frac{1}{k_2-k_1}$$

Therefore, $1/(k-k_1)$ must be linear to $1/[I]^{\frac{1}{n}}$, and this treatment will be more reasonable than that described above, since no approximation is contained. The k_1 value, however, has not yet been obtained, since direct determination of k_1, which must be determined without any ion even such as buffer salts, is very difficult. Indirect determination of k_1 will be tried.

TABLE I

Values[1] *of* k_2 *and* K *for Several Anions*

Anion	n	k_2 (min^{-1}.)	K	Enzyme concentration. (*mg.* Protein-N/*ml.* $\times 10^3$)
Chloride	1	1.7	0.0877	2.10
Sulfate	2	1.7	0.121	0.984
Phosphate (dibasic)	2	1.4	0.0772	1.05
SDS[2]	1	1.7	65.4	2.49
Succinate	2	1.7	0.137	2.10
Citrate	3	0.91	0.157	2.10
Sulfate[3]	2	2.0	0.0689	1.0
Sulfate[3]	2	2.5	0.0392	2.0
Sulfate[3]	2	2.0	0.0301	5.0

All anions were added to the enzyme solution as sodium salts.

1) Calculated by the Equation 8.

2, 3) Enzyme preparation and experimental conditions were the same as in the previous paper (*1*).

3) Experimental data were reported in the previous paper (*1*).

tion that instabilization of sweet potato β-amylase by the action of ion is due only to its charge (*1*). In other words, the amylase combined with an ion with 'one' unit of charge at a certain site of the molecule, will be inactivated by heat at the same rate independent of the species of ions.

Values of K were different for different ions. This may explain the different rates of inactivation in the presence of different ions of same valence, such as halides, which had been reported in the previous paper (*1*). Moreover, it is interesting that K values for different concentrations of the enzyme in the presence of same ion, were different. This may correspond to the relationship between the enzyme concentration and the heat inactivation rate constant (*2*). This problem may be worth while investigating.

Sodium laurylsulfate (SDS), which is known as protein denaturant, increased the rate of heat inactivation of the amylase in the same manner as other neutral salts. Since SDS had almost no effect upon the amylase activity at room temperature at such low concentration as used in this experiment, it appears to act merely as univalent ion. This may suggest also that the ionic charge will contribute to the denaturing action of SDS. And it should be noted that K value of SDS is exceptionally large.

Ionic strength appeared not to participate in these relationships.

Since all these neutral salts are almost without effect upon the amylase activity at room temperature under these experimental conditions, the mechanism of inactivation in the presence of these salts will essentially be that of heat inactivation.

From the results of this work, it may be considered that the charge of ion causes some change in the condition of the charge on the amylase molecule, so that the stability of enzyme protein changes, and that the charge of 'one' site on the amylase protein plays a key role in the stability of the enzyme and possibly in the enzymatic activity.

In the range of higher concentration of ions, the rate of inactivation of amylase decreased as were seen in the case of sulfate and citrate. In general, enzymes are stable in the presence of high concentration of neutral salts, especially sulfate (*5*). Lauffer (*6*) observed that the rate of urea denaturation of tobacco mosaic virus was increased in the presence of lower concentration of salts, but decreased in the presence of higher concentration of them. It is probable that the effects of ions of high concentration on the protein denaturation are different from that of lower concentration.

SUMMARY

The influences of several inorganic and organic neutral salts upon the heat inactivation of sweet potato β-amylase were investigated at various concentration.

From the relationships between the concentration of anions and the rate constant of inactivation, it was assumed that sweet potato β-amylase combined with an anion with 'one' unit of charge, was much more

unstable and was inactivated by heat much faster than free enzyme. And this action of ionic charge appeared to be non-specific to the kind of ions tested.

From these observations, it was assumed also that a certain condition of charge on 'one' site of the amylase protein molecule was essential for stability and possibly for enzymatic activity of the enzyme.

The author wishes to express his appreciation to Prof. S. Akabori and K. Okunuki, Faculty of Science, Osaka University, for their interest and encouragement. Particularly he is indebted to Prof. S. Ono and Dr. K. Hiromi, College of Agriculture, University of Osaka Prefecture, for theoretical discussions, and to Miss S. Amagase for her technical assistance.

REFERENCES

(*1*) Nakayama, S., and Kono, Y., *J. Biochem.*, **45**, 243 (1958)

(*2*) Nakayama, S., and Kono, Y., *J. Biochem.*, **44**, 25, (1957)

(*3*) Balls, A. K., Walden, M. K., and Thompson, R. R., *J. Biol. Chem.*, **173**, 9 (1948)

(*4*) Somogyi, M., *J. Biol. Chem.*, **195**, 19 (1952)

(*5*) Haurowitz, F., "Chemistry and Biology of Proteins," Academic Press, New York, p. 3 (1950)

(*6*) Lauffer, M. A., *J. Am. Chem. Soc.*, **65**, 1793 (1943)

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Enzymatic Studies on Pyridoxine Metabolism

V. Enzymatic Reduction of Isopyridoxal*,**

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(Received for publication, December 16, 1960)

Recently it was reported from this laboratory that in bakers' yeast there is a triphosphopyridine nucleotide-specific pyridoxine dehydrogenase which catalyzes the oxidation-reduction of the hydroxymethyl group at position 4 of pyridoxine (*1*). A partially purified preparation of pyridoxine dehydrogenase, however, was found to reduce isopyridoxal (2-methyl-3-hydroxyl-4-hydroxymethyl-5-formylpyridine) to pyridoxine in the presence of reduced triphosphopyridine nucleotide. It was of interest to determine that the oxidation-reduction of the hydroxymethyl groups at positions 4 and 5 was catalyzed by two distinct enzymes.

Previously Snell and Rannefeld (*2*) found that isopyridoxal could partially replace pyridoxine as a growth factor for *Saccharomyces carlsbergensis*, suggesting that isopyridoxal was converted to pyridoxine, and then to pyridoxal phosphate.

This papar describes a partial purification and some properties of an enzyme from bakers' yeast which catalyzes the reduction of isopyridoxal to pyridoxine. This enzyme will be designated as isopyridoxal reductase on the basis of its action mechanism.

MATERIALS AND METHODS

Isopyridoxal was kindly supplied by Dr. Esmond E. Snell. TPN,* TPNH, and ATP were purchased from Sigma Chemical Company, Missouri, and glucose-6-phosphate and DPN from Nutritional Biochemicals Corporation, Ohio. A pressed cake of bakers' yeast was obtained from Oriental Yeast Company, Osaka. Brewers' yeart was kindly given by Kirin Breweries, Amagasaki.

Glucose-6-phosphate dehydrogenase was prepared from brewers' yeast according to the procedure described by Kornberg and Horecker (*3*). Yeast pyridoxal kinase was purified by the method of Hurwitz (*4*).

Pyridoxine phosphate oxidase was purified from *Pseudomonas aeruginosa* as follows. Ten g. of lyophilized cells of *Pseudomonas aeruginosa* were ground with 3 g. of quartz sand, and extracted with 100 ml. of cold deionized water. After standing for one hour, the mixture was centrifuged at $15{,}000 \times g$ for 20 minutes. To the supernatant 2 ml. of 10% streptomycin sulfate were slowly added. The resulting precipitate was centrifuged off. 43.4 g. of solid ammonium sulfate were added to the supernatant, and the resulting precipitate collected by centrifugation was dissolved in 10 ml. of deionized water. Then this enzyme solution was dialyzed against 100 volumes of 0.005 *M* potassium phosphate buffer, pH 7.4, for 3 hours. Alumina gel Cγ was stirred into the dialyzed enzyme solution at a gel/protein ratio of 0.2, and the mixture was adjusted to pH 5.5 with *N* acetic acid. After 15 minutes, the gel was collected by centrifugation, and eluted with 10 ml. of 0.1 *M* potassium phosphate buffer, pH 7.6. Fifteen- to twenty-fold purification over the crude extract was thus achieved.

Determination of Pyridoxine—Pyridoxine was determined as pyridoxal phosphate by the following pro-

* This work was presented at the 33rd Annual Meeting of the Japanese Biochemical Society (Tokyo) in October-November, 1960.

** This investigation was supported by a grant for scientific research from the Ministry of Education.

* The following abbreviations are used; TPN, TPNH, oxidized and reduced triphosphopyridine nucleotide; DPN, DPNH, oxidized and reduced diphosphopyridine nucleotide; ATP, adenosine triphosphate.

cedure. (a) Pyridoxine was converted to pyridoxine phosphate by pyridoxal kinase and ATP; (b) pyridoxine phosphate was then oxidized to pyridoxal phosphate by pseudomonad pyridoxine phosphate oxidase; (*3*) the resulting pyridoxal phosphate was assayed by the apotryptophanase method (*5*, *6*).

A sample containing less than 1 mμmole of pyridoxine, 20 μmoles of potassium phosphate buffer, pH 6.9, 1.0 μmole of $MgSO_4$, 2 μmoles of ATP and pyridoxal kinase,* in a total volume of 0.6 ml., were incubated for 60 minutes at 31.5°C. The reaction was terminated by heating the mixture at 100°C for 2 minutes. After cooling, 0.1 ml. of 0.5 *M* K_2HPO_4 and 0.1 ml. of pyrdoxine phosphate oxidase were added, and the mixture was shaken at 37°C for 30 minutes. The enzyme was inactivated by immersing the vessel in a boiling water bath for 30 seconds. The pyridoxal phosphate thus formed was determined by the apotryptophanase method.

The formation of pyridoxal phosphate from pyridoxine was linear to a pyridoxine concentration of 1 mμmole, as shown in Fig. 1. Therefore, for

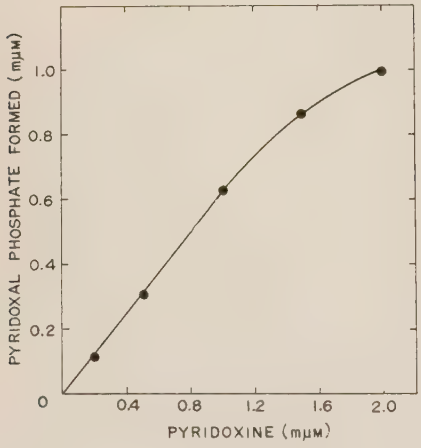

Fig. 1. Assay of pyridoxine. Pyridoxine was converted to pyridoxal phosphate by the procedure described in the text.

the assay of pyridoxine, an aliquot of the incubation mixture containing less than 1 mμmole of pyridoxine was taken. The pyridoxine phosphate oxidase activity was inhibited slightly by the pyridoxal phosphate formed; so a correction was made for this. Pyridoxal kinase, pyridoxine phosphate oxidase and apotryptophanase were free of isopyridoxal reductase activity.

* At least 20 Hurwitz's units of the kinase were used throughout the following experiments.

Assay of Isopyridoxal Reductase—The isopyridoxal reductase activity was assayed in the system described below. One hundred mμmoles of isopyridoxal, 40 μmoles of potassium phospate buffer, pH 7.4, 0.5 μmole of TPN, 4 μmoles of glucose-6-phosphate, 2 mg. of glucose-6-phosphate dehydrogenase and an isopyridoxal reductase preparation, in a volume of 0.8 ml., were incubated for 60 minutes at 31.5°C. The reaction was terminated by heating the mixture for 2 minutes in a boiling water bath. An appropriate aliquot of the incubation mixture was taken for the determination of pyridoxine. A unit of isopyridoxal reductase was defined as the amount of enzyme which formed 1.0 mμmole of pyridoxine in 60 minutes at 31.5°C.

Protein was determined by the method of Kalcker (*7*).

RESULTS

Paper Chromatography of Reaction Product—One half μmole of isopyridoxal was incubated at pH 7.4 with an autolysate of bakers' yeast

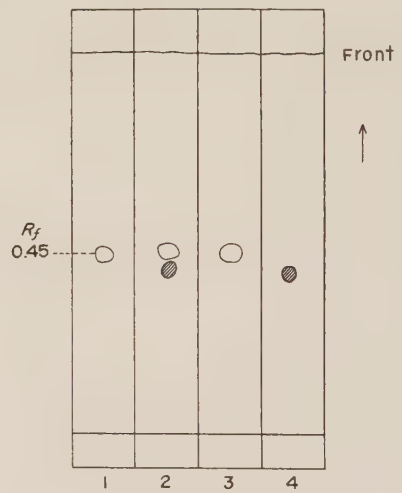

Fig. 2. Paper chromatogram of the reaction mixture.

1) Autolysate+isopyridoxal
2) Autolysate+isopyridoxal+TPNH
3) Autolysate+isopyridoxal+DPNH
4) Autolysate+pyridoxine

Spots were seen under ultra-violet light or visualized by spraying with diazotized sulfanilic acid.

and a TPNH-generating system for 3 hours at 37°C. Then 4 volumes of ethanol were added to stop the reaction. The supernatant

obtained after centrifugation was concentrated *in vacuo*. The concentrate was applied to Toyo Roshi No. 51 filter paper, and chromatographed in *n*-butanol, acetic acid, water (4 : 1 : 1). As shown in Fig. 2, spots corresponding to isopyridoxal and pyridoxine appeared. Omission of the TPNH-generating system from the incubation mixture or its replacement by a DPNH-generating system did not result in pyridoxine formation. No pyridoxal was formed under these conditions. Further proof that pyridoxine is the direct product in this system will be described later.

Purification of Isopyridoxal Reductase—One hundred and forty g. of air-dried bakers' yeast were autolyzed in 440 ml. of 0.1 *M* $NaHCO_3$ for one hour at 37°C and for two and half hours at 30°C. The supernatant obtained after centrifugation had a volume of 245 ml. All subsequent operations were carried out near 0°C. To each 100 ml. of the autolysate were added 31.3 g. of solid ammonium sulfate. After 40 minutes, the mixture was centrifuged and the precipitate discarded. To the supernatant solution an additional 10.1 g. of solid ammonium sulfate per 100 ml. were added and the resulting precipitate was dissolved in 28 ml. of water. This fraction was then dialyzed against 3 l. of deionized water for 3 hours, and the dialyzed enzyme was treated with alumina gel C_γ at pH 6.0 at a gel/protein ratio of 0.5 (w/w). After 15 minutes' stirring, the gel was collected by brief centrifugation. The gel was eluted by stirring it with one half the original volume of 0.2 *M* potassium phosphate buffer, pH 7.4, and after 15 minutes the mixture was again centrifuged to remove the gel. Enzyme recovery by this treatment was about 160 per cent of that of the ammonium sulfate fraction. This may have been due to the removal of contaminating TPNH oxidase activity by this treatment. The gel eluate was brought to pH 4.8 by dropwise addition of *N* acetic acid, and was allowed to stand for 30 minutes. The bulky precipitate was centrifuged off, and the supernatant was readjusted to pH 7.4 with *N* KOH. The

Table I

Purification of Isopyridoxal Reductase and Comparison of Activity with Pyridoxine Dehydrogenase

For the assay of isopyridoxal reductase, see "Materials and Methods."

Assay for pyridoxine dehydrogenase was carried out as follows: the reaction mixture containing, in 1.0 ml., 10 μmoles of pyridoxine, 1.0 μmole of TPN, 100 μmoles of sodium carbonate buffer, pH 9.0, and the enzyme preparation was incubated for 20 minutes at 37°C. Pyridoxal was determined as its phenylhydrazone[1].

Fraction	Step	Specific Activity		Ratio
		Isopyridoxal reductase	Pyridoxine dehydrogenase[2]	
		units/mg. protein	units/mg. protein	
A	Autolysate	2.96	2.50	1.18
B	$(NH_4)_2SO_4$ farctionation (0.5–0.65 saturation)	3.00	18.8	0.16
C	Alumina gel C_γ eluate	31.8	65.0	0.49
D	Acid treatment, pH 4.8	72.5	37.5	1.93
	$(NH_4)_2SO_4$ fractionation			
	0–0.5 saturation	0.96	1.26	0.76
	0.65–0.8 saturation	3.40	13.3	0.26
	Heat denaturation, 50°C, 2 minutes	2.41	38.0	0.06

1) Dr. H. Wada; personal communication.
2) A unit of enzyme activity was defined as the amount of enzyme which causes the formation of 1.0 mμmole of pyridoxal in 10 minutes.

acid-treated enzyme had full activity.

A summary of the purification procedure is given in Table I.

During the purification of isopyridoxal reductase, fractions were also assayed for pyridoxine dehydrogenase to determine whether these two activities resided on one enzyme or not. As seen clearly from the last column of Table I, the ratio of the two activities was very different in different preparation. Isopyridoxal reductase was resistant to acid treatment, while pyridoxine dehydrogenase activity was rather sensitive to it. The reverse situation was observed on heat treatment at 50°C for 3 minutes.

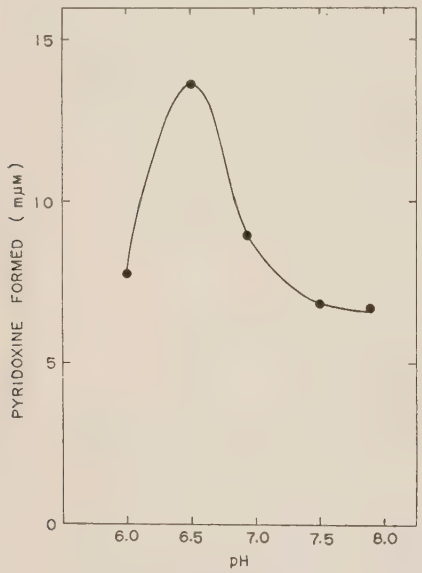

Fig. 3. pH dependence of the reaction.

Reaction mixture contained, in a volume of 0.6 ml., 40 μmoles of potassium phosphate buffer, 100 mμmoles of isopyridoxal, 0.2 μmole of TPNH and 0.8 mg. of Fraction D protein. Incubation for 20 minutes at 37°C.

The fraction precipitating between 0.65 and 0.8 saturation of ammonium sulfate had a higher specific isopyridoxal reductase activity. The total units, however, were much less than in the fraction precipitating between 0.5 and 0.65 saturation.

pH Dependence of Reaction—The pH dependence of the reduction of isopyridoxal was studied by incubating isopyridoxal with Fraction D protein and TPNH. As shown in Fig. 3, isopyridoxal reduction had a rather sharp pH optimum at pH 6.5. In experiments in which a TPNH-generating system was used instead of TPNH, incubations were carried out at a higher pH.

Table II

Identification of Reaction Product

100 mμmoles of isopyridoxal were incubated with 6.7 mg. of Fraction B protein with TPNH-generating system as described in "Materials and Methods." Aliquots of the incubation mixture assayed for pyridoxine and pyridoxal. Two aliquots were incubated with pyridoxal kinase[1] and ATP to form pyridoxine phosphate from pyridoxine. One of the aliquots was then oxidized by pyridoxine phosphate oxidase while another was not.

Expt. No.	Pyridoxine-PO_4 oxidase	Pyridoxal-PO_4 formed
		mμmole
1	+	19.3
2	−	0.0

1) Pyridoxal kinase preparation had no pyridoxine phosphate oxidase activity.

Identification of Reaction Product—To determine whether pyridoxine is the sole reaction product or whether some pyridoxal is also formed by contaminating pyridoxine dehydrogenase, the experiment shown in Table II was performed. No pyridoxal phosphate was formed in an incubation mixture which had not been treated with pyridoxine phosphate oxidase. This indicates that only pyridoxine is formed under these incubation conditions.

Cofactor Requirements in Reduction of Isopyridoxal—A preliminary experiment using paper chromatography shows that TPNH rather than DPNH was required for the reduction of isopyridoxal to pyridoxine. Table III shows an experiment on the cofactor specificity of the reaction. DPNH was about 6 per cent as active as TPNH in this experiment. It should be noted that pyridoxine dehydrogenase shows an absolute requirement for TPN (*1*).

Table III

Cofactor Specificity for Reduction of Isopyridoxal to Pyridoxine

1.8 mg. of Fraction C protein was used. Incubation conditions were same as those described in "Materials and Methods" except that incubation was carried out at 37°C.

As a DPNH-generating system, 17 μmoles of ethanol, 0.5 μmole of DPN and 0.88 mg. of crystalline alcohol dehydrogenase were used.

System		Pyridoxine formed
		mμmole
Complete		59.4
//	—TPNH-generating system	0.0
//	+DPNH-generating system, instead of TPNH-generating system	3.6

Time Course of Reaction—The formation of pyridoxine from isopyridoxal as a function of time is seen in Fig. 4. An initial lag period was noted in pyridoxine formation.

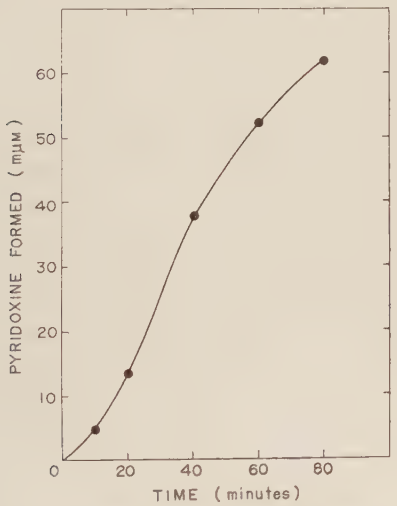

Fig. 4. Time course of the reaction.

Incubation mixture contained, in a volume of 1.9 ml., 80 μmoles of potassium phosphate buffer, pH 7.4, 200 mμmoles of isopyridoxal, 0.5 μmole of TPN, 12 μmoles of glucose-6-phosphate, 6 mg. of glucose-6-phosphate dehydrogenase, 1.8 mg. of Fraction C protein.

Appropriate aliquot of the incubation mixture was taken at the time indicated.

This might be due to the inadequate generation of TPNH. The reaction proceeded in an essentially linear fashion for the first 60 minutes, after which formation of pyridoxine was retarded.

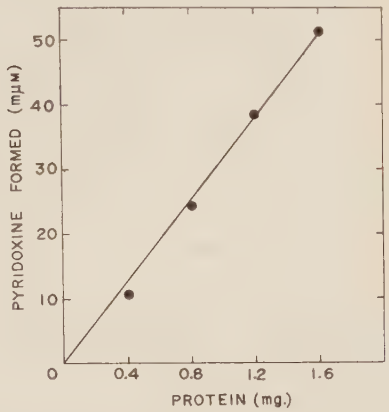

Fig. 5. Effect of the enzyme concentration on the reaction rate.

40 μmoles of potassium phosphate buffer, pH 7.4, 100 mμmoles of isopyridoxal, 0.5 μmole of TPN, 4 μmoles of glucose-6-phosphate, 2 mg. of glucose-6-phosphate dehydrogenase and varying amount of Fraction C protein, in a volume of 1.0 ml., were incubated for 60 minutes at 37°C.

Effect of Enzyme Concentration on Reaction Rate—The conversion of isopyridoxal to pyridoxine as a function of the enzyme con-

Table IV

Distribution of Isopyridoxal Reductase Activity

Organism	Isopyridoxal reductase[1]
Bakers' yeast	14.1
Brewers' yeast	0
Escherichia coli	13.4
Lactobacillus casei	0
Streptococcus faecalis	0
Pseudomonas aeruginosa	0
Sacchromyces carlsbergensis	+
Rat liver[2]	0
Rat kidney[2]	0

1) mμmole of pyridoxine formed per hour per 10 mg. dry weight at 37°C.

2) 0.2 ml. of 20% homogenate was used.

centration is plotted in Fig. 5. A proportionality was observed between pyridoxine formation and the enzyme concentration.

Distribution of Enzyme Activity—Table IV shows data on the distribution of isopyridoxal reductase in several microorganisms and rat tissues. Only bakers' yeast and *Escherichia coli* had marked isopyridoxal reductase activity. It must be recalled that there are considerable differences in its distribution from that of pyridoxine dehydrogenase (*1*).

DISCUSSION

From the data described above, it is clear that the oxidation-reduction of the hydroxymethyl group at position 5 of pyridoxine is catalyzed by a different enzyme from that catalyzing the oxidation-reduction at position 4.

Snell and Rannefeld (*2*) reported that isopyridoxal could replace as a growth factor for *Saccharomyces carlsbergensis*. Isopyridoxal had no effect on the growth of *Streptococcus faecalis* and *Lactobacillus casei*. These findings are consistent with our result (Table IV). It is also shown that isopyridoxal has a slight growth-promoting effect in rats (*2*, *8*). However, we were unable to show any detectable reduction of isopyridoxal by homogenates of rat liver or kidney.

Recently, Rodwell, Volcani, Ikawa and Snell (*8*) found that pyridoxine was oxidized to isopyridoxal by bacteria isolated from soil by the enrichment culture technique, and showed isopyridoxal to be one of the naturally occurring substances having a vitamin B_6 activity. However, detailed data on their study of this reaction mechanism are not yet available.

SUMMARY

Bakers' yeast was found to catalyze the reduction of isopyridoxal to pyridoxine in the presence of TPNH. A partial purification of isopyridoxal reductase from bakers' yeast is described. The pH optimum of the reaction is at 6.5. DPNH was much less effective than TPNH in the reduction of isopyridoxal. From a comparison of the activities of the two enzymes, it is concluded that isopyridoxal reductase is a distinct enzyme from pyridoxine dehydrogenase. The distribution of isopyridoxal reductase activity in some organisms and rat tissues was studied.

REFERENCES

(*1*) Morino, Y., and Sakamoto, Y., *J. Biochem.*, **48**, 733 (1960)
(*2*) Snell, E. E., and Rahnefeld, A. N., *J. Biol. Chem.*, **157** 475 (1945)
(*3*) Kornberg, A., and Horecker, B. L., in "Methods in Enzymology" (Colowick, S. P. and Kaplan, N. O. Eds.), Vol. **1**, Academic Press Inc., New York, p. 323 (1955)
(*4*) Hurwitz, J., *J. Biol. Chem.*, **205**, 935 (1953)
(*5*) Wada, H., Morisue, T., Sakamoto, Y., and Ichihara, K., *J. Vitaminol.*, **3**, 183 (1957)
(*6*) Nakahara, I., Morino, Y., Morisue, T., and Sakamoto, Y., *J. Biochem.*, **49**, 339 (1961)
(*7*) Kalcker, H., *J. Biol. Chem.*, **167**, 461 (1947)
(*8*) Rodwell, V. W., Volcani, B. E., Ikawa, M., and Snell, E. E., *J. Biol. Chem.*, **233**, 1548 (1958)

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Enzymatic Studies on Pyridoxine Metabolism

VI. Pyridoxine Metabolism in Pregnancy*, **

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(Received for publication, December 20, 1960)

It had been reported that there is an increased excretion of xanthurenic acid in the urine of pregnant subjects (*1*). This fact was supposed to indicate that pregnancy causes vitamin B_6 deficiency in which a disturbance in tryptophan metabolism is known to occur, leading to an increased excretion of xanthurenic acid or other tryptophan metabolites in the urine. It was in fact demonstrated that the administration of vitamin B_6 to pregnant subjects lowered xanthurenic acid excretion to a normal level (*2*).

A recent study in this laboratory has shown that in mammalian tissues pyridoxal phosphate is mainly formed from pyridoxine phosphate (*3*), so that pyridoxine phosphate oxidase serves as a key enzyme. Therefore, it was interesting to study vitamin B_6 metabolism in pregnancy to gain further insight on the role of pyridoxine phosphate oxidase in pyridoxine utilization.

This paper reports data on the correlation of the pyridoxal phosphate content and pyridoxine phosphate oxidase activity during pregnancy and embryonal development.

MATERIALS AND METHODS

Animals used were non-pregnant, mature rats, pregnant rats in early, middle, and late stages of pregnancy, middle and late stage embryos, and suckling rats of various ages. The stages of pregnancy were as follows; the early stage lasted till the 7th day after the appearance of erythrocytes in vaginal smears: the middle stage, from the 8th to the 14th day; the late stage, from the 15th to the 21st day.

Animals were sacrificed by a blow on the head and exsanguinated by cutting the juglar vein. Their livers were immediately removed and weighed in portions on a torsion balance. The tissue was homogenized with 4 volumes of cold deionized water in a glass homogenizer. The homogenate thus obtained was divided into two portions, one of which was used for the assay of enzyme activity. The other was re-homogenized briefly with 4/5 volume of 10% metaphosphoric acid and placed in a water bath at 85°C for 15 minutes. After cooling, the denatured protein residue was centrifuged off. The supernatant was adjusted to pH 8.0 with 50% KOH and diluted with cold deionized water so as to give a 5% extract with respect to the original tissue weight. 1.0 ml. of the extract was employed for the assay of pyridoxal phosphate, using apotryptophanase prepared from dried cells of *E. coli* (K 12) (*4*).

Pyridoxine phosphate oxidase activity was measured as follows. The reaction mixture containing in 1.0 ml., 100 μmoles of potassium phosphate buffer, pH 8.0, 0.5 μmole of pyridoxine phosphate and 0.2 ml. of the liver homogenate, was incubated for 30 minutes at 37°C. The reaction was stopped by immersing the tubes in a boiling water bath for one minute. The amount of pyridoxal phosphate formed was assayed with apotryptophanase (*4*) and by the color developed with dinitrophenylhydrazine***. Apotryptophanase preparations made according to the above method had a considerable pyridoxine phosphate oxidase activity, which interferred with the measurement of the pyridoxine phosphate oxidase activity in the liver homogenate. This interference was prevented by prolonging the isoelectric treatment at pH 4.4, which was therefore usually carried out overnight without dialysis. This resulted in complete inactivation of pyridoxine phosphate oxidase in the apotryptophanase prepara-

* This work was presented, in part, at the 7th Kinki Local Meeting of the Japanese Biochemical Society in May, 1960, and 22nd Kinki Local Meeting of the Japanese Obstetrical and Gynecological Society in May, 1960.

** This work was supported by a research grant from Wachsman Foundation.

*** Dr. H. Wada; personal communication

tions as well as in a complete dissociation of pyridoxal phosphate.

Pyridoxal kinase was assayed as follows. The incubation mixture containing in 1.0 ml., 2.0 μmoles of pyridoxal, 2.0 μmoles of ATP, 1.0 μmole of $MgSO_4$, 50 μmoles of potassium phosphate buffer, pH 6.0, and 0.1 ml. of a 20% homogenate was incubated for one hour at 37°C. Pyridoxal phosphate formed was determined using apotryptophanase.

A preliminary experiment revealed that an enzymatic dephosphorylation of pyridoxal phosphate is catalyzed by acid and alkaline phosphatases in an non-specific manner*. Therefore, *p*-nitrophenyl phosphate (*5*) was employed as a substrate for the measurement of pyridoxal phosphate phosphatase activity.

The crude calcium salt of pyridoxine phosphate was prepared by phosphorylation of pyridoxine with phosphorus oxychloride (*6*). Its purification was achieved on an IRC-50 resin column (*7*).

Results and Discussion

1. Pyridoxal phosphate level in the liver.

As was shown in Table I, larger amounts of pyridoxal phosphate were found in livers of pregnant rats than in those of non-pregnant mature rats. Also it can be seen in the table that the pyridoxal phosphate content of fetal livers was very low and increased gradually with development, to reach the level of non-pregnant, mature rats two weeks after delivery.

Table I

Pyridoxal Phosphate Content of Liver from Control, Pregnant Rats, their Embryos and Suckling Rats

Livers from	Number of animals	Pyridoxal phosphate content (average values)
		in μg. per g. of wet tissue
Control rats	12	5.7
Pregnant rats		
Early stage	4	6.6
Middle stage	6	7.0
Late stage	5	7.2
Embryos in middle and late stage	28	1.9
Suckling rats		
One day old	5	1.9
Two days old	7	3.2
Thirteen days old	7	4.3

2. Pyridoxine phosphate oxidase activity.

Fig. 1 shows that pyridoxine phosphate oxidase activity in livers during fetal development was less than one-fourth of that in mature, nonpregnant animals, but increased daily during the postnatal stage to mature level. In the figure is also included an average value of the liver pyridoxine phosphate oxidase activity of pregnant rats. This is considerably higher than that of nonpregnant control animals.

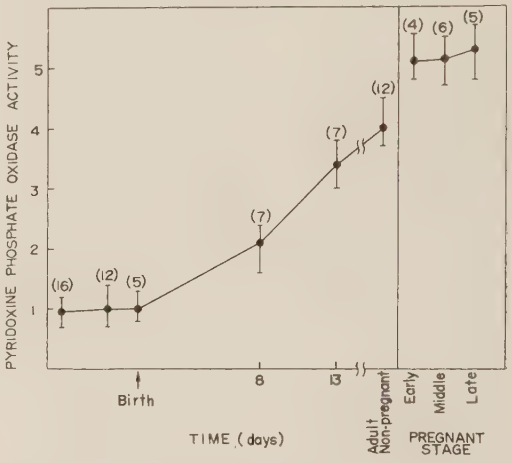

Fig. 1. Change of rat liver pyridoxine phosphate ovidase activity with development.

Pyridoxine phosphate oxidase activity was expressed as mμmoles of pyridoxal phosphate formed by 50 mg. of liver in 30 minutes at 37°C in the standard assay system. The number of rats used for experiment was shown in the parenthesis. The mean values were represented by closed circles and the range was indicated by a vertical bar which passes through each mean value.

It is very reasonable here to consider the influence of pregnancy and development upon the activity of the related enzymes, *i. e.*, pyridoxal kinase and phosphatase. In fact, no change was detected in the activity of these enzymes during pregnancy or development.

The parallerism observed between changes

* Unpublished observation

in the pyridoxal phosphate content and pyridoxine phosphate oxidase activity would give further support to the idea of the important role of pyridoxine phosphate oxidase in pyridoxal phosphate formation. It might also reflect a dynamic aspect of the dependency of embryonal development upon maternal vitamin B_6 metabolism. Recently Wachstein *et al.* (*8*) reported a decrease in the pyridoxal phosphate content of serum and its elevation in cordal blood in pregnant women and from a vitamin B_6 load test concluded that pregnancy causes a relative pyridoxine deficiency. Our results, together with their finding, would indicate that the mechanism for pyridoxal phosphate production is stimulated at least in livers of pregnant subjects in response to embryonal development.

It was also of interest to test the effects of progesterone, estradiol and the gonadotropic hormone upon the liver pyridoxal phosphate level and pyridoxine phosphate oxidase activity. Table II shows that administration of progesterone, estradiol and a mixture of these hormones to nonpregnant mature female rats did not result in any change in either the pyridoxal phosphate content or pyridoxine phosphate oxidase activity, while injection of the gonadotropic hormone resulted in changes similar to those in pregnancy.

The results described above clearly show that the pyridoxal phosphate level in rat liver increases as a consequence of an elevation of pyridoxine phosphate oxidase activity in pregnancy and also under the influence of the gonadotropic hormone.

Effects of other hormones on vitamin B_6 metabolism are also under investigation.

TABLE II

Effects of Administration of Various Hormones on Liver Pyridoxal Phosphate Content and Pyridoxine Phosphate Oxidase Activity

Hormon injected[1]	Number of animals	Pyridoxal[2] phosphate content	Pyridoxine[3] phosphate oxidase activity
None[4]	6	5.6	3.9
Estradiol benzoate[5]	8	5.3	4.0
Progesterone[6]	7	5.2	3.4
Estradiol benzoate plus progesterone[7]	4	5.1	4.2
Gonadotropic hormone[8]	11	7.0	5.3

1) The indicated amount of hormone was daily injected subcutaneously for five days prior to experiments.

2) in μg. per g. of wet tissue

3) in mμmole pyridoxal phosphate formed by 50 mg. of liver in 30 minutes at 37°C in the standard assay system

4) 0.5 ml. of propylene glycol per day

5) 0.2 mg. of estradiol benzoate suspended in 0.5 ml. of propylene glycol per day

6) 0.2 mg. of Progenin (Teikoku Hormone MFG. CO. LTD., Tokyo) suspended in 0.5 ml. of propylene glycol per day

7) 5) plus 6)

8) 10 rabbit units of a mixture of gonadotropic hormones from anterior pituitary gland and chorion membrane dissolved in 0.5 ml. of saline per day

SUMMARY

The pyridoxal phosphate content of nonpregnant mature rats, pregnant rats, their embryos and sucklings and the pyridoxine phosphate oxidase activity were determined in the livers. The levels were high in the livers of pregnant rats and low in those of embryos. After delivery, the levels gradually reached normal levels in livers of both postnatal sucklings and their mothers. Injection of the gonadotropic hormone resulted in levels similar to those in pregnancy with respect to the pyridoxal phosphate content and pyridoxine phosphate oxidase activity.

REFERENCES

(*1*) Wachstein, M., and Gudaitis, A., *J. Lab. Clin. Med.*, **42**, 98 (1953)

(*2*) Zartman, E. R., Barnes, A. C., and Hicks, D. J., *Am. J. Obst. Gynecol.*, **70**, 645 (1955)

(*3*) Wada, H., Morisue, T., Nishimura, Y., Morino, Y., Sakamoto, Y., and Ichihara, K., *Proc. Japan Acad.*, **35**, 299 (1959)

(*4*) Wada, H., Morisue, T., Sakamoto, Y., and

Ichihara, K., *J. Vitaminol.*, **3**, 183 (1957)
(5) Fukuyama, and Yamashita, *Koshueisei-in Kenkyu Hokoku*, **2**, 5 (1952)
(6) Heyl, D., Luz, E., Harris, S. A., and Folkers, K., *J. Am. Chem. Soc.*, **73**, 3430 (1951)
(7) Peterson, E. A., and Sober, H. A., *J. Am. Chem. Soc.*, **76**, 169 (1954)
(8) Wachstein, M., Keliner, J. D., Ortiz, J. M., *Proc. Soc. Exptl. Biol. Med.*, **103**, 350 (1960)

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Enzymatic Studies on Pyridoxine Metabolism

VII. The Influence of Fat and Fatty Acid Administration upon Pyridoxine Metabolism*, **

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It had been reported by Mannering *et al.* (*1*) that rats fed on a diet rich in fat exhibited an unusually high requirement for riboflavin. Later Kotake *et al.* (*2*) observed that when tryptophan was given to rats fed on a high fat diet or administered with a large dose of various fatty acids, there was a marked increase in urinary xanthurenic acid excretion. Charconnet-Harding *et al.* (*3*) also reported on the increased excretion of some tryptophan metabolites including xanthurenic acid in the urine of rats fed on a vitamin B_2 deficient diet. It was recognized by several workers (*4*) that the abnormal increase of xanthurenic acid excretion in the urine was due to vitamin B_6 deficiency. Thus a close relationship between the roles of vitamins B_2 and B_6 in tryptophan metabolism could be readily inferred. Recently Kotake *et al.* (*5*) found a lowered level of pyridoxal phosphate in the livers of rats after administration of fatty acids. However, no definite explanation could be given for the mechanism by which this decrease in the pyridoxal phosphate content occurred.

Recent findings in this laboratory have shown that in mammalian tissues, pyridoxal phosphate is formed from pyridoxine *via* pyridoxine phosphate, so that pyridoxine phosphate oxidase serves as a key enzyme (*6*). It was also demonstrated that this enzyme is a flavoprotein (*7*).

In the present work the authors examined the effect of fatty acid administration and of a diet rich in fat on the activity of pyridoxine phosphate oxidase and other enzymes concerned with pyridoxal phosphate formation in rat liver, in an effort to clarify the mechanism of the effect of fatty acids.

MATERIALS AND METHODS

Young albino rats weighing 120 to 140 g. were used as experimental animals. They were fed on Oriental chow MF *ad libitum*. The food was removed about 12 hours before the onset of the experiments. The fatty acids employed were sodium butyrate, sodium caproate, palmitic acid, stearic acid and linoleic acid. 0.3 g. each of fatty acid or its salt dissolved or suspended in 1.0 ml. of water was given orally to the rats. Control animals were given 1.0 ml. of water. At various time intervals after administration of the fatty acids the rats were killed by decapitation. The livers were removed, weighed and immediately homogenized with 4 volumes of ice cold deionized water in a Potter-Elvehjem type homogenizer. The homogenate was divided into two portions. The one was used for measurement of the pyridoxal phosphate content and the other for the assay of pyridoxine phospha te oxidase, pyridoxal phosphate phosphatase and pyridoxal kinase as described in the previous paper (*8*).

The high fat diet consisted of butter (30 per cent), casein (20 per cent), starch (33 per cent), McCollum salt (6 per cent), agar (4 per cent) and the following vitamins (in μg. per g. of diet): thiamine hydrochloride, 3.3; nicotinic acid, 10; calcium pantothenate, 13.3; choline hydrochloride, 166; inositol, 333; riboflavin, 6.6; *p*-aminobenzoic acid, 200; pyridoxine hydrochloride, 3.3. The control diet contained the same components as the high fat diet except for butter

* This work was presented at the 33rd Annual Meeting of the Japanese Biochemical Society (Tokyo) in October-November, 1960.

** This work was supported by a research grant from Wachsman Foundation.

(10 per cent) and starch (55 per cent). Rats were fed with these diets *ad libitum*. A vitamin B_2 deficient diet was prepared according to the description by Kotake *et al.* (*9*).

Results and Discussion

Liver Pyridoxal Phosphate Content and Pyridoxine Phosphate Oxidase Activity of Rats Fed on a High Fat Diet—Both the pyridoxal phosphate content and the pyridoxine phosphate oxidase activity were determined at weekly intervals. They were found to decrease gradually and at the end of the fourth week were about 50 per cent of the control values (Fig. 1). The change in pyridoxine phosphate oxidase activity seemed to precede that of the pyridoxal phosphate. This suggests that pyridoxal phosphate formation decreased as a consequence of decreased pyridoxine phosphate oxidase activity.

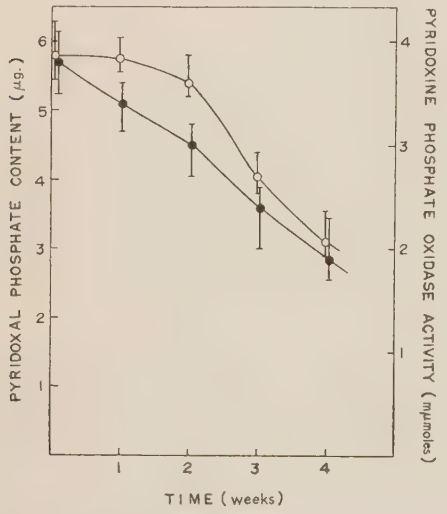

Fig. 1. Variation with time of liver pyridoxal phosphate content and pyridoxine phosphate oxidase activity of rats fed on a high fat diet.

Open symbols represent pyridoxal phosphate content, and closed symbols pyridoxine phosphate oxidase activity.

To test the effect of supplementation of vitamin B_2 on liver pyridoxal phosphate and pyridoxine phosphate oxidase activity in rats fed on a high fat diet, the control and high fat diet groups were subdivided into two further groups and rats in one of each two subgroups received a daily subcutaneous injection of 200 μg. of FMN during the last week of the experiment. At the end of the third week, animals were sacrificed and their pyridoxal phosphate content and pyridoxine phosphate oxidase activity measured. As shown in Table I, FMN administration prevented the depression both of the liver pyridoxal phosphate content and the pyridoxine phosphate oxidase activity of rats fed on a high fat diet. This indicates that the ingestion of much fat causes a relative deficiency of vitamin B_2, resulting in a decrease of pyridoxine phosphate oxidase, a flavin enzyme. This again results in a depression of pyridoxal phosphate formation.

Liver Pyridoxal Phosphate Content and Pyridox-

Table I

Decrease in Pyridoxal Phosphate Content and Pyridoxine Phosphate Oxidase Activity in Rat Liver Fed on a High Fat Diet for 3 Weeks and its Prevention by Simultaneous Administration of FMN

	No. of animals	Pyridoxal phosphate	Pyridoxind[1] phosphate oxidase activity	Pyridoxal[2] kinase activity
		μg./g. liver		
Control	10	5.8±0.5	3.8±0.5	1.24±0.2
〃 , FMN administered[3]	9	5.4±0.7	3.8±0.3	1.23±0.16
High fat diet	9	4.0±0.4	2.4±0.3	1.21±0.21
〃 , FMN administered[3]	8	5.2±0.5	3.5±0.5	1.24±0.17

1) mμmoles of pyridoxal phosphate formed from pyridoxine phosphate by 50 mg. of liver in 30 minutes.

2) mμmoles of pyridoxal phosphate formed from pyridoxal by 50 mg. of liver in 30 minutes.

3) 200 μg. of FMN were injected daily subcutaneously.

ine Phosphate Oxidase Activity of Rats Administered with Various Fatty Acids—The liver pyridoxal phosphate content and pyridoxine phosphate oxidase activity were measured at intervals after oral administration of sodium butyrate. As shown in Fig. 2, both the pyridoxal phosphate content and the pyridoxine phosphate oxidase activity began to fall one hour later and reached minimal values after about 4 hours. They later returned to normal levels. In these animals pyridoxal kinase and pyridoxal phosphate phosphatase were also assayed but no change was found in their activities. Therefore, it was concluded that pyridoxine phospaate oxidase activity was depressed initially due to fatty acid administration and subsequently pyridoxal phosphate formation decreased.

Next the effects of various fatty acids were examined. Table II shows that caproic

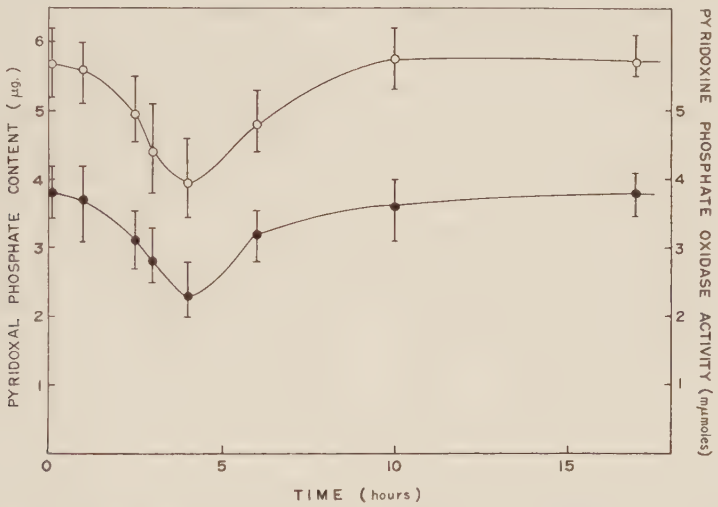

Fig. 2. Variation of liver pyridoxal phosphate content and dyridoxine phosphate oxidase activity with time after oral administration of sodium butyrate.

Open symbols represent pyridoxal phosphate content, and closed symbols pyridoxine phosphate oxidase activity.

Table II

Effect of Administration of Various Fatty Acids on Liver Pyridoxal Phosphate Content and Pyridoxine Phosphate Oxidase Activity

Fatty acid administered	No. of animals	Pyridoxal phosphate content		Pyridoxine phosphate oxidase activity	
			Vitamin B_2 injected[1]		Vitamin B_2 injected[1]
Control	10	5.8±0.5		3.8±0.5	
Butyrate	8	4.1±0.5	5.6±0.4	2.3±0.5	3.8±0.3
Caproate	5	3.1±0.6	4.9±0.4	1.4±0.4	3.7±0.5
Palmitate	5	4.6±0.5	4.8±0.6	2.9±0.3	3.9±0.4
Stearate	5	3.9±0.7	4.5±0.6	2.0±0.6	3.2±0.5
Linoleate	7	6.2±0.5	6.0±0.4	3.4±0.4	3.6±0.4

1) 100 μg. of riboflavin were subcutaneously injected at the time of fatty acid administration.

acid, palmitic acid and stearic acid exerted effect similar to that of butyric acid. However, linoleic acid had no significant effect on the pyridoxal phosphate content and pyridoxine phosphate oxidase activity. The difference between the effects of fatty acids helps to clarify the mechanism of action of fatty acids, for it has recently been reported that linoleic acid is metabolized in a different way from other saturated fatty acids (*10*). Most saturated fatty acid are degraded by the well-known β-oxidation pathway. However, the metabolism of linoleic acid has been reported to involve pyridine nucleotide rather than a flavin coenzyme.

Effect of Vitamin B_2 Supplementation on Decrease in Pyridoxal Phosphate Content and Pyridoxine Phosphate Oxidase Activity—As in the case of fat feeding, the effect of butyrate administration was prevented by the simultaneous injection of riboflavin (Table II). This finding strengthens the idea that fatty acids exert their effects upon a flavoprotein, pyridoxine phosphate oxidase, by disturbing the overall vitamin B_2 metabolism in the liver in an unknown way. Actually, the activity of xanthine oxidase, a flavin enzyme, was also found to be depressed under similar conditions. Furthermore, the addition of FMN *in vitro* appeared to restore the lowered pyridoxine phosphate oxidase activity and, on the other hand, addition of FAD restored the depressed xanthine oxidase activity. This finding emphasizes the fact that fatty acid administration causes a relative vitamin B_2 deficiency.

The total flavin content of the livers of control and sodium butyrate-treated rats is shown in Table III. A slight decrease in the flavin content in livers of butyrate-administered rats was observed.

It was of interest to examine whether the liver pyridoxal phosphate content and pyridoxine phosphate oxidase activity of rats fed on a vitamin B_2 deficient diet would actually decrease. Rats were kept on a vitamin B_2 deficient diet for two weeks and their livers were assayed for pyridoxal phosphate and pyridoxine phosphate oxidase. Table IV shows the results which are quite similar to those obtained when fat and fatty acids were administered to the rats. Therefore, it is highly probable that the heavy ingestion of fat or fatty acids causes a relative vitamin B_2 deficiency in which the activity of pyridoxine phosphate oxidase, a flavin enzyme, decreases, causing a subsequent depression of pyridoxal phosphate formation. Although the present findings are not sufficient to demonstrate the exact mechanism of the action of fatty acids in their interaction with vitamin B_2 metabolism, it should be emphasized that at least the casual mechanism of the effects of fatty acids on vitamin B_6 metabolism was clearly shown.

TABLE III

Total Vitamin B_2 Content in Rat Liver 4 Hours after Administration of Sodium Butyrate

	No. of animals	Vitamin B_2 content
		μg./g. liver
Control rat	8	28.3±3.17
Butyrate adiministered rat	10	24.7±3.1

Vitamin B_2 was converted to lumiflavin; the fluorescence of the latter was measured fluorometrically by the method described by Fukumori (*11*).

TABLE IV

Liver Pyridoxal Phosphate Content and Pyridoxine Phosphate Oxidase Activity of Livers of Rats Fed on a Vitamin B_2 Deficient Diet

	No. of animals	Pyridoxal phosohate content	Pyridoxine phosphate oxidase activity	Pyridoxal kinase activity
		μg./g. liver		
Control	8	5.8±0.5	3.8±0.5	1.24±0.15
Vitamin B_2 deficient	11	5.0±0.51	3.0±0.45	1.23±0.15

There is a possibility that the effect of fatty acids is due to the direct interaction with flavin enzymes. However, this will be excluded since pyridoxine phosphate oxidase was not inhibited *in vitro* by these fatty acids.

SUMMARY

Weekly determination on rats fed on a high fat diet for 4 weeks revealed a gradual decrease in the pyridoxal phosphate content and pyridoxine phosphate oxidase activity. This was prevented by the simultaneous administration of FMN. Similarly, oral administration of butyrate caused a decrease in the pyridoxal phosphate content and pyridoxine phosphate oxidase activity. Caproate, stearic acid and palmitic acid had similar effects. The decrease was prevented by the simultaneous injection of riboflavin. In these cases the depression of pyridoxal phosphate content was probably due to a decrease in the pyridoxine phosphate oxidase activity.

REFERENCE

(*1*) Mannering, G. J., Lipton, M. A., and Elvehjem, C. A., *Proc. Soc. Exptl. Biol. Med.*, **46**, 100 (1941)
(*2*) Kotake, Y. Jr., and Inada, T., *Proc. Japan Acad.*, **28**, 65 (1952)
(*3*) Charconnet-Harding, F., Dalgliesh, C. E., and Neuberger, A., *Biochem. J.*, **53**, 513 (1953)
(*4*) Lepkovsky, S., Roboz, E., and Hangen-Smit, A. J., *J. Biol. Chem.*, **149**, 195 (1943)
(*5*) Nakai, S., *J. Wakayama Med. Soc.*, **8**, 33 (1957)
(*6*) Wada, H., Morisue, T., Nishimura, Y., Morino, Y., Sakamoto, Y., and Ichihara, K., *Proc. Japan Acad.*, **35**, 299 (1959)
(*7*) Morisue, T., Morino, Y., Sakamoto, Y., and Ichihara, K., *J. Biochem.*, **48**, 28 (1960)
(*8*) Nakahara, I., Morino, Y., Morisue, T., and Sakamoto, Y., *J. Biochem.*, **49**, 339 (1961)
(*9*) Kotake, Y., in "Jikken Kagaku Koza" (Kotake, M., Ed.), Vol. **25**, Maruzen, Tokyo, p. 351 (1958)
(*10*) Lynn, W. S., and Brown, R. H., *Arch. Biochem. Biophys.*, **81**, 353 (1959)
(*11*) Fukumori, T., *Vitamins* (Japan), **20**, 225 (1960)

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Studies on Streptolysin S' Formation by Streptococcal Ghosts

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It has been found that the formation of a hemolysin, streptolysin S'*, is greatly stimulated by the addition of oligonucleotides with high guanylic acid content (*1*). The mechanism of its formation, however, remains to be elucidated.

Since it was shown by Hosoya *et al.* (*2*) and Bernheimer (*3*) that streptolysin can be formed by resting streptococci, some investigations on the mechanism of the toxin formation have been reported with the system.

It was anticipated that studies with a subcellular system might provide further insight into the mechanism and attempts have been made to get subcellular preparations capable of forming streptolysin.

Maruyama, Sugai and Egami (4, 5) reported that streptococcal protoplasts can form streptolysin. The author has found that streptococcal ghosts, obtained by lysing protoplasts in hypotonic media, have an ability to form streptolysin and the preparation is suitable to study the mechanism of its formation. The purposes of this paper are to show that streptolysin S' can be formed by streptococcal ghosts and to define the conditions necessary for the toxin formation by ghosts. Some data bearing on the mechanism of the toxin formation are also presented. Preliminary reports of this paper have already been published (*6*, *7*).

* During the study, Dr. T. Hayashi of Hokkaido University found that a streptolysin produced in the presence of the active fraction from nucleic acid differs from streptolysin S designated by Todd (*13*). Therefore the author used the term streptolysin S' as used by several authors such as Younathan and Barkulis (*8*).

MATERIALS AND METHODS

Organism—Streptococcus pyogenes strain S8 (a group A streptococcus) was used. Culture was maintained on blood agar slants.

Culture Technique—Five ml. of a peptone-horse heart broth (a modified Todd-Hewitt medium) was inoculated. After incubation for 15 hours at 37°C, the culture was transferred into 100 ml. of the same broth and incubated for 6 hours at 37°C. Cells were collected by centrifugation and washed twice with sodium phosphate saline (pH 7.0).

Preparation of the Active Fraction—The active fraction (AF) was prepared by ECTEOLA-cellulose column chromatography of pancreatic ribonuclease core of yeast ribonucleic acid by the method of Tanaka (*1*).

Preparation of a Lytic Enzyme—The lytic enzyme used for breaking cell walls was prepared from a phage lysate of a group C streptococcus by the method of Krause (*9*). The phage lysate was obtained according to Maxted (*10*).

Preparation of Ghosts—Protoplasts were prepared with the use of the lytic enzyme in 0.5 *M* succinate as reported previously (*5*). To obtain ghosts, protoplasts were suspended in a medium of pH 7.0 containing 0.2 *M* succinate, 0.01 *M* $MgSO_4$ and 20 μg. per ml. of crystalline pancreatic deoxyribonuclease. A highly viscous clump appeared when protoplasts were suspended in the medium. After 15 minutes' incubation at 37°C, the clump was dispersed by the action of deoxyribonuclease and the resulting homogeneous suspension was centrifuged at 10,000 r.p.m. for 10 minutes in the cold. The pellet was washed with ice-cold 0.2 *M* succinate (pH 7.0) containing 0.01 *M* $MgSO_4$ and again separated by centrifugation. Under an electron microscope, the pellet was found to consist of ghosts and no intact cells were recognized.

Determination of Streptolysin—After ghosts were spun down from the reaction mixture, the hemolytic activity in the supernatant was determined by the two-fold dilution method with the use of a freshly

prepared, 3% rabbit erythrocytes suspension. The hemolytic unit (H. U.) is the amount of hemolysin which will lyse half the erythrocytes contained in 1 ml. of phosphate saline (pH 7.0) in 2 hours at 37°C.

Determination of Protein and Nucleic Acid—Protein and nucleic acid fraction were obtained according to the method of Schneider (*11*). Ribonucleic acid (RNA) was determined with the orcinol reagent and protein by the method of Lowry (*12*).

Amino Acids—An acid-hydrolyzed casein was a Difco product (Bacto Casamino acid). Purified amino acids were obtained from Ajinomoto Co.

RESULTS

Composition and Stability of Ghosts—Electron micrographical examination showed that ghosts consisted of transparent membrane to which were attached some electron-dense components (*6*). The protein and RNA content of ghosts were 38 and 36 per cent of intact cells, respectively.

Ghosts could be washed by a medium containing 0.01 *M* $MgSO_4$ below 5°C without significant loss of activity, but when they were washed by 0.06 *M* phosphate buffer, pH 7.0, they lost their activity (Table I). When

TABLE I

Effect of Washing Medium on Streptolysin Forming Activity

Washing Medium	H. U.
1. 0.06 *M* phosphate buffer, pH 7.0	0
2. 1. containing 0.01 *M* $MgSO_4$	100
3. 0.2 *M* Na-succinate, pH 7.0	130
4. 3. containing 0.01 *M* $MgSO_4$	190
5. 4. containing 0.02 *M* thioglycolic acid	130

Ghosts were washed by these media below 5°C, transferred into the standard incubation medium containing 0.2 *M* succinate and incubated for 60 minutes.

ghosts were treated at 37°C, it was necessary to contain 0.2 *M* succinate (pH 7.0) in the medium in order to retain streptolysin-forming activity. Malonate could replace succinate.

Ghosts could not be stored frozen, even if 0.2 *M* succinate was in the medium.

Incubation Medium—In the following incubation medium ghosts could produce an appreciable amount of streptolysin: 0.2 *M* sodium succinate, 0.002 *M* $MgSO_4$, 0.03 *M* KH_2PO_4, 0.005 *M* maltose, 0.02 *M* thioglycolic acid and 100 μg. per ml. of AF, pH 7.0. Ghosts were suspended in the medium at a concentration of 2 mg. dry weight per ml. and incubated at 37°C.

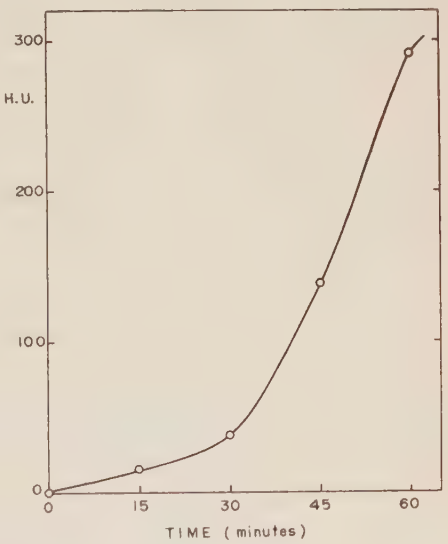
TABLE II

Requirements for Streptolysin Formation by Ghosts

Condition	Streptolysin formed (H.U.)
Complete system	370
Minus succinate	0
Minus thioglycolate	34
Minus maltose	34
Minus active fraction	0

Incubations were for 60 minutes.

The requirement for these substances is shown in Table II and a typical time course under the complete system is given in Fig. 1.



Time course of streptolysin formation by ghosts.

The amount of streptolysin produced by ghosts was 10–20 per cent of that produced by intact cells under these conditions.

Effect of Sulfhydryl Compounds—As thioglycolic acid was necessary for streptolysin for-

mation by ghosts, other sulfhydryl compounds were tested and also found to be effective (Table III).

TABLE III

Effect of SH Compounds

SH compound added	Streptolysin formed (H.U.)
None	98
Thioglycolate	780
Cysteine	312
Glutathione	171

The concentration of SH compounds was 0.02 *M* and incubations were carrid out for 60 minutes.

Effect of Chloramphenicol and An Amino Acid Analogue—Younathan and Barkulis (*8*) observed that antibiotics and some of most potent inhibitor among amino acid analogues and the inhibition was reversed by phenylalanine. The effect of these substances was tested with ghosts. The results were similar to those obtained with intact cells (Table IV, V, VI).

TABLE IV

Inhibition of Streptolysin Formation by Chloramphenicol

Concentration of Chloramphenicol	H. U.
0 *M*	390
1×10^{-6}	256
1×10^{-5}	185
1×10^{-4}	99
1×10^{-3}	0

Incubation were for 60 minutes.

TABLE V

Inhibition of Streptolysin Formation by β-DL-phenylserine

Concentration of β-DL-phenylserine	H. U.
0 *M*	530
1×10^{-4}	21
5×10^{-4}	25
1×10^{-3}	19
3×10^{-3}	16

Incubations were for 60 minutes.

amino acid analogues inhibited the production of streptolysin S′ by intact cells. According to their results, β-DL-phenylserine was the

TABLE VI

Reversal of DL-phenylserine Inhibition of Streptolysin Formation by L-phenylalanine

Concentration of phenylalanine added	H. U.
0 *M*	29
1×10^{-6}	144
1×10^{-5}	305
1×10^{-4}	350
1×10^{-3}	530
No phenylserine nor phenylalanine	530

Concentration of phenylserine was $1\times10^{-3}M$. Incubations were for 60 minutes.

Effect of Amino Acids—As indicated in Table VII, the addition of an acid-hydrolyzed casein enhanced the production of streptolysin. In order to examine whether

TABLE VII

Effect of an Acid-hydrolyzed Casein on Streptolysin Formation

Concentration of Acid-hydrolyzed Casein	Ghosts	Intact cells (H.U.)
0 mg./ml.	206	4,400
0.5	284	120,000
1	284	60,000
2	430	
5	512	5.000
10	2048	4,100
20	2048	3,400

Incubations with intact cells were carried out under conditions similar to those used for incubations with ghosts. All incubations were carried out for 60 minutes.

the effect of the acid-hydrolyzed casein was due to amino acids or other components which might contaminate the preparation, a number of mixtures of purified amino acids of different composition were tested for their

Table VIII

Effect of Amino Acids on Streptolysin Formation by Ghosts

Amino acids added	H. U.
1. None	45
2. 17 Amino acids[1)]	330
3. 2 minus pro	74
4. 2 minus his	64
5. Casamino acid 10 mg./ml.	210

1) 17 amino acids were gly, L-pro, L-glu, DL-met, L-lys, L-leu, DL-val, DL-ala, DL-thr, L-ser, L-tyr, L-his, L-asp, L-phe, L-arg, DL-try and L-cystine, the concentration of each amino acid was 0.25 mg./ml. Incubations were for 60 minutes.

Table IX

Effect of Amino Acid Mixtures on Streptolysin Formation by Intact Cells

Amino acids added	H. U.
None	1850
17 amino acids	2700
// minus gly	1540
// minus pro	11200
// minus glu	5550
// minus met	1500
// minus ser	4600
// minus tyr	2660
// minus his	16400
// minus asp	2600
// minus phe	1700
// minus arg	1750
// minus try	2500
// minus lys	2660
// minus leu	1100
// minus val	790
// minus ala	2560
// minus thr	610
// minus cystine	1020

The composition of 17 amino acids was same as described in Table VIII and the concentration of amino acid was 0.025 mg./ml. Incubations were carried out for 60 minutes under similar conditions but without succinate.

effect. As shown in Table VIII, streptolysin formation by ghosts was enhanced when 17 amino acids were contained in the mixture.

It was noticed that the addition of an amino acid mixture also enhanced streptolysin formation by intact cells (Table VII). When intact cells were used, the concentration of amino acid mixture for the optimum production of streptolysin was much lower (Table VII). It was also found that certain amino acid mixtures increased streptolysin formed by intact cells, while others decreased it (Table IX).

DISCUSSION

The data presented here show that streptolysin S' appears by a synthetic process wich resembles protein synthesis in several respects.

The subcellular system reported here has a perfect reproducibility and seems to be suitable preparation for the study of the mechanism of streptolysin formation. In this system, that it is ghosts and not remaining intact cells if any in the preparation that are concerned in streptolysin formation is evident, because the system requires succinate. The succinate does not enhance streptolysin formation by intact cells. The function of succinate on streptolysin formation by ghosts has not yet been elucidated, but it seems to have the effect of stabilizing ghosts rather than to be an energy source, because malonate can replace succinate. The concentration of succinate used in the system is not high enough to support protoplasts of this bacterium.

It was found that the addition of thioglycolic acid increased streptolysin formation by intact cells as well as by ghosts and the effect was marked when the concentration of cells was low. This fact as well as the fact that streptolysin was produced anaerobically in Thunberg tubes suggests that the formation proceeded under reducing conditions.

Amino acid mixtures were found to stimulate streptolysin S' formation. The reason why the effect was not observed by Younathan and Barkulis may be that they used intact cells and either composition

or concentration of their amino acid mixture was not suitable. Ghosts have a smaller amino acid pool than intact cells (less than 30 per cent by the author's observation) and they may depend on exogeneous amino acids for streptolysin formation. Whether the effect of amino acid mixture is due to amino acid incorporation into streptolysin or to other indirect causes remains to be elucidated.

The present experiment does not provide any suggestion to the role of AF in streptolysin formation. However, by using the system described in this paper, the elucidation of the problem may be possible.

SUMMARY

1. Streptococcal ghosts obtained by lysing protoplasts in hypotonic media were shown to form streptolysin S′ when 0.2 *M* succinate was present in the incubation medium.

2. Thioglycolic acid, cysteine and glutathione stimulated streptolysin formation by ghosts.

3. Chloramphenicol and β-DL-phenylserine inhibited streptolysin formation by ghosts and the latter inhibition was reversed by phenylalanine.

4. An acid-hydrolyzed casein and a mixture of 17 purified amino acids enhanced streptolysin formation by ghosts. Certain amino acid mixtures were found to enhance streptolysin formation by intact cells.

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REFERENCES

(*1*) Tanaka, K., *J. Biochem.*, **45**, 109 (1958)
(*2*) Hosoya, S., Hayashi, T., Homma, Y., Egami, F., Shimomura, M. and Yagi, Y., *Japan. J. Exptl. Med.*, **20**, 27 (1949)
(*3*) Bernheimer, A. W., *J. Exptl. Med.*, **90**, 37 (1949)
(*4*) Maruyama, Y., Sugai, S., and Egami, F., *Nature*, **811**, 832 (1959)
(*5*) Maruyama, Y., Sugai, S., and Egami, F., *Z. Allgem. Mikrobiol.* **1**, 25 (1960)
(*6*) Sugai, S., and Egami, F., *Proc. Japan Acad.*, **36**, 141 (1960)
(*7*) Sugai, S., and Egami, F., *J. Biochem.*, **48**, 777 (1960)
(*8*) Younathan, E. S., and Barkulis, S. S. *J. Bacteriol.*, **74**, 151 (1957)
(*9*) Krause, M., *J. Exptl. Med.*, **108**, 803 (1958)
(*10*) Maxted, W. R., *J. Gen. Microbiol.*, **16**, 584 (1957)
(*11*) Schneider, W. C., *J. Biol. Chem.*, **161**, 293 (1945)
(*12*) Lowry, O. H., Rosebrough, J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.*, **193**, 263 (1951)
(*13*) Todd, E. W., *J. Path. Bact.*, **47**, 423 (1938)

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An Improved Method for the Preparation of Triphosphopyridine Nucleotide from Pig Liver

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(Received for publication, November 14, 1960)

Recently, Pontis *et al.* (*1*) reported that neutral aqueous ethanol is able to elute several nucleoside diphosphate sugars such as uridine diphosphate glucose from charcoal, after washing the charcoal with EDTA** solution. Application of this EDTA ethanol elution technique to LePage's method (*2*) for the isolation of TPN revealed that this modification simplifies considerably the isolation procedure***. A typical experiment is presented below.

All the procedures were carried out at 25—30°C except special steps indicated. Six kg. of hog liver (from 4 animals) was packed in ice and brought to the laboratory. The livers were minced and added at once to 9 liters of boiling water. After keeping over 90°C for 15 minutes the mixture was cooled to about 40°C by dipping the container in cold water. The mixture was then filtered through gauze layers using suction and the residue was again extracted with 9 liters of boiling water. The extracts obtained were combined (18 liters). One hundred and twenty g. of activated charcoal (Wako: Shirasagi A) was added to the extract and the suspension was shaken for 15 minutes and settled for two hours. After the supernatant fluid was siphoned off, the charcoal was collected using suction. The filter cake was washed with 1.8 liters of water, 1.8 liters of 0.01 *M* EDTA (pH 8.0–8.5) and 1.8 liters of water successively, in which the charcoal was well suspended and then collected using suction. The washed cake was treated with 1.2 liters and 0.8 liters of 50% ethanol successively in the same manner for the elution of TPN. The eluates were combined and poured into four volumes of 99% ethanol (or two volumes of acetone), and the pH of the mixture was adjusted to 2.0 (with thymol blue) by the addition of 6 *N* HNO_3. Flocculent precipitate was formed immediately. After settling overnight at 5-10°C the clear supernatant fluid was siphoned off and the precipitate was collected, washed three times with absolute ethanol and dried in vacuum. Grey powder containing about 5% TPN was obtained. The powder was dissolved in 50 ml. of water and adjusted to pH 7.0 (with phenol red) by the cautious addition of aqueous ammonia. Resulting brown solution was passed through a Dowex-1 X8 formate column (10 cm. in height, 2.5 cm. in diameter), and then the column was washed with 100 ml. of water. The elution of nucleotides from the column was carried by a gradient elution technique in which 800 ml. of water was used as a mixer content and a reservoir was placed by 400 ml. of 1 *N* formic acid and 800 ml. of 4 *N* formic acid successively. The elution was carried out in a flow rate of 5 ml. per minute. Each 10 ml. frac-

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** EDTA: ethylenediaminetetraacetate, DPN and TPN: di- and triphosphopyridine nucleotide.

*** Application of the same technique to the isolation of DPN and Coenzyme A from baker's yeast proved unsatisfactory.

tions were collected and assayed for the absorption at 260 mμ. The fractions showing high ultraviolet absorption were assayed by cyanide test (*3*) for pyridine coenzymes. The first and the third peak were positive to this test*. The latter cyanide positive fractions were combined and pooled in the cold. The pooled eluate was added to 9 volumes of 99% ethanol (or four volumes of acetone) and pH was adjusted to 2.0 (with thymol blue) by the addition of 6 *N* HNO_3. After the suspension was settled within several hours in the cold, resulted white precipitate was collected by centrifugation in the cold, washed three times with absolute ethanol and dried in vacuum. Two hundred and fifty mg. of greyish white powder was obtained. TPN content of this powder was 82% based on isocitric dehydrogenase assay (*3*) without correction for moisture, and perfectly free from DPN on yeast alcohol dehydrogenase assay (*3*).

* The first, second and the third peak were DPN, adenosine monophosphate and TPN respectively.

REFERENCES

(*1*) Pontis, H. G., Cabib, E., and Leloir, L. F., *Biochim. et Biophys. Acta*, **26**, 146 (1957)

(*2*) LePage, G. A., and Mueller, G. C., *J. Biol. Chem.*, **180**, 975 (1949)

(*3*) Ciotti, M. M., and Kaplan, N. O., "Methods in Enzymology", Academic Press Inc., New York, Vol. III, p. 890 (1957)

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Cofactor Requirements for Anthranilate Oxidase

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Received for publication, December 3, 1960)

In a preliminary note (*1*), we have reported a partial purification of anthranilate oxidase from a soil bacterium adapted to anthranilate and on some properties of this enzyme. We have also found that the enzyme activity was completely lost by ammonium sulfate fractionation and fully restored by the addition of a TPNH-generating system (containing glucose-6-phosphate dehydrogenase), reduced glutathione and a boiled extract of *E. coli*, strain B.

The present communication is concerned with further studies on the nature of anthranilate oxidase.

The bacterium used in the present studies was isolated from garden soil and later identified as a strain of *Pseudomonas fluorescens**, which was tentatively named strain B-23.

Extraction and protamine treatment of the enzyme(s) was carried out by the same procedure as described previously with the exception that albumin was omitted from the extraction medium (*1*). The preparation was further fractionated with ammonium sulfate into two parts, precipitating between 0.33–0.45 (Fraction A-1) and 0.45–0.55 (Fraction B-1) saturation. Fraction A-1 was adsorbed onto alumina Cγ and eluted with 0.1 *M* phosphate buffer, pH 7.5 (Fraction A-2). Fraction B-1 was adsorbed on calcium phosphate gel and eluted with 0.1 *M* phosphate buffer, pH 7.5 (Fraction B-2). These two fractions (Fraction A-2 and Franction B-2) were used in combination in most of the experiments reported here.

It was found that previously reported active principle of the boiled extract of *E. coli* (*1*) could be replaced by DPN. This caused us to re-examine the specificity of pyridine nucleotides in the oxidation of anthranilate in more detail using another reduced pyridine nucleotide-generating system, glucose-glucose dehydrogenase. Table I compared the activities of the two nucleotides, DPN and TPN, coupled with glucose dehy-

Table I

Nucleotide Specificity in Anthranilate Oxidation

Nucleotide	pH	Oxygen uptake[1]		
		Anthranilate		△
		+	−	
DPN	7.0	24.7	4.5	20.2
	7.5	30.0	3.3	26.7
	8.0	10.2	3.7	6.5
TPN	7.0	15.3	3.3	12.0
	7.5	21.3	3.6	17.0
	8.0	20.7	4.1	16.6
None	7.5	4.1	2.1	2.0

The reaction mixture contained; 2 μmoles of glutathione, 5 μmoles of anthranilate, 0.1 μmole of DPN or TPN, 1 μmole of ferrous sulfate, 100 μmoles of glucose, 900 units of glucose dehydrogenase, 200 μmoles of Tris-HCl buffer, 0.2 ml. of Fraction A-2, 0.2 ml. of Fraction B-2, and water to a final volume of 2.0 ml. Incubations were carried out at 30°C.

1) μl. per 10 minutes.

* We are greatly indebted to Dr. M. Yamanaka, Department of Bacteriology, Osaka Medical College, Osaka, for identifying the bacterium.

drogenase at different pH values. It is clear that both nucleotides are effective in the oxidation of anthranilate. However, in the observed pH range, the reaction rate is more affected by changes in pH with DPN than with TPN. For example, at pH 8.0 the activity with TPN is 94 per cent, while with DPN it is only 24 per cent, of the activity at pH 7.5. The difference in the effect of pH on these nucleotides is not clearly understood at present. However it is unlikely that it is simply due to a difference in the pH optimum of glucose dehydrogenase with each nucleotide, because the pH optimum of glucose dehydrogenase is above 9.0 with both nucleotides (*2*).

Table II

Stoichiometry with Pyridine Nucleotides

Nucleotide	Anthranilate added[1]	Oxygen uptake	Ammonia liberated[2]	β-Ketoadipate fromed[3]
	μmoles	μmoles	μmoles	μmoles
DPN	5.0	10.1	4.5	4.8
TPN	5.0	9.3	4.0	4.6

The reaction system was as described in Table I. The fractions were washed twice with cold water to minimize their ammonia contents before being eluted from the gels. The Tris-HCl buffer used was pH 7.5. Incubations were carried out at 30°C for 150 minutes.

1) During the incubation, the absorption of anthranilate at 310 mμ completely disappeared.

2) Determined by the modified method of Lubochinsky and Zalta (*3*).

3) Determined by catalytic decarboxylation of 4-aminoantipyrine (*4*).

To study the specificity of pyridine nucleotides further, a stoichiometric analysis of the reaction with the separate nucleotides was performed. As shown in Table II, whether DPN or TPN was used, there was an equimolar formation of β-ketoadipic acid and ammonia. This also shows that there is no difference in the specificity of these nucleotides in anthranilate oxidation. It must be added that, in the absence of a reduced pyridine nucleotide-generating system or pyridine nucleotides, there was no liberation of ammonia from anthranilate.

Besides the inhibitors reported previously (*1*), metal chelating agents, such as *o*-phenanthroline and α,α'-dipyridyl, are potent inhibitors of the reaction (*5*). The inhibition by *o*-phenanthroline was reversed by the addition of ferrous ion but of other metallic ions. Furthermore the enzyme activity at the final stage of purification (Fraction A-2 plus Fraction B-2) was greatly (more than 50 per cent) stimulated by the addition of ferrous ion. Since the concentration of the inhibitor used (1×10^{-3} *M*) does not significantly affect the activity of pyrocatechase, these facts strongly suggest that ferrous ion is involved in the oxidation of anthranilate to catechol.

As mentioned above, the anthranilate oxidase preparation could be separated into two fractions (Fraction A-2 and Fraction B-2). Separately, these showed only slight activity towards anthranilate even in the presence of indispensable cofactors. However a combination of these two fractions had great enzyme activity, as shown in Table III.

Table III

Separation of Anthranilate Oxidase

Fraction	Oxygen uptake[1]		
	Anthranilate		Δ
	+	−	
Fraction A-2	5.0	1.9	3.1
Fraction B-2	5.7	0.7	5.0
Combined	23.8	1.6	22.2

The reaction system was as described in Table I. The Tris-HCl buffer used was pH 7.5. Incubations were carried out at 30°C.

1) μl. per 10 minutes.

Although the significance of these two components is not clear at present, it is interesting to note both fractions are only formed inducibly.

REFERENCES

(*1*) Hosokawa, K., Nakagawa, H., and Takeda, Y.,

J. Biochem., **48**, 155 (1960)
(*2*) Strecker, H.J., "Methods in Enzymology" (Colowick and Kaplan, eds.) Vol. **1**, Academic Press, New York, p. 339, (1955)
(*3*) Lubochinsky, B., and Zalta, J., *Bull. soc. chim. biol.*, **36**, 1363 (1954)
(*4*) Sistrom, W.R., and Stanier, R.Y., *J. Bacteriol.*, **66**, 404 (1953)
(*5*) Higashi, T., and Sakamoto, Y., *J. Biochem.*, **48**, 147 (1960)

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Steroidsulfatase in the Liver of *Charonia lampas*

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(Received for publication, December 6, 1960)

A large marine gastropod, *Charonia lampas* (*Tritonalia sauliae*), has been shown to be an excellent source of various sulfatases by Soda, Egami and their coworkers. However, the existence of steroidsulfatase found in related animals such as *Helix pomatia* (*1*) and *Patella vulgata* (*2*) has not so far been investigated in the organism.

The present note describes the steroidsulfatase in the partially purified sulfatase preparation from the liver of *Charonia lampas*.

EXPERIMENTAL

Substrates—Dehydroepiandrosterone sulfate (DHAS) was kindly supplied by Dr. K. S. Dodgson. Scymnol sulfate extracted from shark bile was kindly supplied by Dr. L. F. Fieser.

Enzyme Solution—Partially purified enzyme solution (Preparation B) prepared according to Takahashi (*3*) was used.

Reaction Mixture and Experimental Conditions—The activity of steroidsulfatase was measured in the presence of 0.1 *M* sodium acetate (pH 4.0–6.0) and 0.1 *M* tris-maleate buffer (pH 5.5–7.0) with 0.0002 *M* DHAS or scymnol sulfate. Final volume of reaction mixture was 1 ml. containing 0.1 ml. enzyme solution. Incubation time at 37°C was 1 hour with DHAS and 4 hours with scymnol sulfate, respectively.

Enzyme Assay—Enzyme assay was carried out by the method of Dodgson and Spencer (*4*). Enzyme unit is defined as enzyme quantity liberating 1 μmole of sulfate per hour under optimum conditions. Specific activity is defined as number of units per mg. protein.

RESULTS

The activities of steroidsulfatase and arylsulfatase of "Preparation B" are summarized in Table I.

Table I

Specific Activities of Steroidsulfatase and Arylsulfatase of "Preparation B"

Steroidsulfatase activity was determined at a final substrate concentration of 0.0002 *M* dehydroepiandrosterone sulfate (DHAS) at stated pH in 0.1 *M* buffers. Incubations were carried out at 37°C for 1 hour. Arylsulfatase activity was determined at a final substrate concentration of 0.01 *M* nitrocatechol sulfate (NCS) at pH 6.0 in 0.02 *M* acetate buffer. Incubation was for 30 minutes at 37°C.

	Arylsulfatase	Steroidsulfatase						
Substrate	NCS	DHAS						
pH	6.0	4.0	4.5	5.0	5.5	6.0	6.5	7.0
Specific activity[1)]	78	5.7	6.6	8.0	8.3	7.5	6.2	5.4

1) Enzyme unit is defined as enzyme quantity liberating 1 μmole of sulfate per hour unders optimum conditions.

Specific activity is defined number of unit per mg. protein.

As shown in Table I, the liver of *Charonia lampas* contains a highly active steroidsulfatase hydrolyzing DHAS. The optimum pH of 5.1–5.5 was obtained. As the sulfate ester bond in scymnol sulfate is attached to an aliphatic side chain (*5*), it is not surprising that it was not hydrolyzed by the enzyme preparation.

SUMMARY

1. The liver of a marine gastropod, *Charonia lampas* contains a highly active

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steroidsulfatase hydrolyzing dehydroepiandrosterone sulfate. The optimum pH of 5.1–5.5 was obtained.

2. Scymnol sulfate was not hydrolyzed by the enzyme preparation.

Authors wish to thank Dr. L. F. Fieser and Dr. K. S. Dodgson, who kindly supplied us scymnol sulfate and dehydroepiandrosterone sulfate, respectively. A part of the expence of this study was defrayed by a grant from the Ministry of Education and from Seikagaku-kenkyusho Ltd., to which their thanks are due. Some of the experiments were carried out in the Marine Biological Station of Nagoya University.

REFERENCES

(*1*) Henry, R., Thevenet, M., and Jarrige, P., *Bull. Soc. Chim. Biol.*, **34**, 896 (1952)

(*2*) Roy, A. B., *Biochim. et Biophys. Acta*, **15**, 300 (1954); Roy, A. B., *Biochem. J.*, **62**, 41 (1956) and Leon, Y. A., Bulbrook, R. D., and Corner, E. D. S., *Biochem. J.*, **75**, 612 (1960)

(*3*) Takahashi, N., *J. Biochem.*, **48**, 508 (1960)

(*4*) Dodgson, K. S., and Spencer, B., "Methods of Biochemical Analysis," ed. by Glick, D., New York, Vol. **IV**, p. 231 (1957)

(*5*) Bergmann, W., and Pace, W. T., *J. Am. Chem. Soc.*, **65**, 477 (1943)